ree PhD Year 2008 Name of Author ENYAKOIT, George Ojula

Cytogenetic Analysis of DNA Copy Number Aberrations in High Malignancy Grade Astrocytomas

A thesis submitted for the degree of
Doctor of Philosophy

By

George Ojula ENYAKOIT

The Galton Laboratory
University College London
4 Stephenson Way, London NW1 2HE

Institute of Neurology

The National Hospital for Neurology and Neurosurgery
(University College London)
Queen Square, London WC1 3BG

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Declaration

I declare that this thesis which is submitted for the award of the degree of Doctor of Philosophy (DPhil) is the culmination of original research which I have personally carried out, and, where necessary with the help of colleagues and supervisors whose contributions are dully acknowledged. Authors of corroborating information and other references are clearly acknowledged in references.

Signed

24 April 08

Date

Principal Supervisor

Professor Sue Povey MD, FMedSci
Haldane Professor of Human Genetics
Galton Laboratory,
Department of Biology,
University College London
Wolfson House
4 Stephenson Way
London NW1 2HE

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ABSTRACT

Astrocytomas are the most common variety of primary tumours of the central nervous system (CNS). The incidence increases with age, peaking in patients aged 65 – 75 years. They are generally unresponsive to treatment, and most patients die within one year of diagnosis. Recent genetic studies of astrocytoma susceptibility syndromes, familial- and sporadic astrocytomas have led to the discovery of many genes and molecular mechanisms underlying astrocytoma oncogenesis. A few of the genes involved in inherited astrocytoma associated-syndromes (e.g., Cowden and Li-Fraumeni syndromes) are also strongly implicated in sporadic astrocytomas. However for several other well characterised genes i.e. those mutated in Tuberous sclerosis (*TSC*) and Neurofibromatosis (*NF*) any evidence for involvement in sporadic astrocytomas is much less clear. The objective of this study was to undertake a genome-wide survey of high malignancy grade astrocytomas with a view to ascertaining the distribution and prevalence of copy number aberrations, search for associations with prognosis and outcomes to treatment, and to discover possible novel pathways of oncogenesis.

Thirty-two high malignancy grade astrocytomas were investigated. Twenty were available as frozen biopsies and 12 as short-term cell cultures. Genomic profiling of all 32, comprising 6 tumours of WHO Grade III and 26 of Grade IV, was achieved by the method of Comparative Genomic Hybridisation onto metaphase chromosomes. 7 of the tumours were investigated by array CGH, with one further investigated by MFISH. Two tumours did not reveal aberrations. For the other 30 tumours, data pooled from a minimum of 10 profiles of each tumour were analysed.

Recurrent DNA copy number gains and losses were detected across the genome. In a number of tumours aberrations spanned loci of established candidate genes previously associated with sporadic astrocytomas, on chromosomes 7, 9p, 10q, 12q, 13q and 17p. In addition over 70% of 'sporadic' tumours appeared to have DNA-copy number aberrations implicating genes with established roles in astrocytoma-susceptibility syndromes. The chromosomal region 9q34 (site of *TSC1*) appeared to be under-represented in 25% of the tumours, while 16p13 (site of *TSC2*) was diminished in ~38%. Similarly, loci for NF1 and NF2 were involved in aberrations in ~10% and 38% of the cases respectively, as were those of PMS2 (22%), APC (19%) and a number of miss-

match repair (MMR) genes. Eight tumours had probable loss at 1p36. Several novel locations were also suggested

An attempt to correlate DNA copy number alterations with survival showed that among patients with grade IV tumours, there were on average far fewer chromosome aberrations per tumour in four of the five patients surviving longer than one year after diagnosis than in those who died before this. There was some suggestion of a worse prognosis in Grade IV tumours with a specific deletion of 1p36, which would agree with a previous report.

The only Grade IV tumour that was studied by all three approaches showed good agreement between array and metaphase CGH. In addition, array CGH revealed small regions of loss in the region of PTEN (CHR 10q) and TP53 (CHR 17p) below the resolution of the metaphase CGH. The M-FISH revealed very large numbers of chromosomes and several translocations, and comparison of the microarray data and the MFISH data suggests possible candidate regions for breakpoints. The data are discussed in the light of previous work and with a view to the possibility that there may be some diversity in the cellular origin of astrocytomas and that haploinsufficiency may play some role in oncogenesis

CHAPTER 1 INTRODUCTION

Brain tumours comprise approximately 5% of all human cancers (ENCR, 2001) of which ~ 70% arise from glial cells and are known as gliomas (Kleihues & Cavenee, 2000). Diffuse astrocytomas, which are the major subset of gliomas derived from astrocytes, account for about 93 - 95% of astrocytomas (Garcia & Fulling, 1985; Forsyth et al., 1993; Szymas et al., 2000; Lewis et al., 2005) while the remaining 5 – 7% constitute a predominantly benign category known as pilocytic astrocytomas (Kleihues & Cavenee, 2000). Based on histological appearances diffuse astrocytomas are graded as low grade (LGAs) (WHO malignancy grade II) or high-grade (HGAs) (WHO grades III & IV). The HGAs have a very poor prognosis, with most patients dying within a year of diagnosis (Sant et al., 2003). However, the biological behaviours of these tumours vary greatly and the rationale behind this thesis is the idea that understanding the genetic factors altered in the tumours may allow a more accurate assessment of prognosis and more effective management options.

1-1 Glial cells and the relationship to astrocytomas

Typically two main types of mature terminally differentiated glial cells, oligodendrocytes and type-1 astrocytes (T1As), are identified in vivo in the adult CNS. Developmentally, they are thought to arise from different precursor cell populations, which define separate glial lineages (Figure 1), with T1As presumed as the sole end-point of differentiation along the T1A lineage. On the other hand, oligodendrocytes arise from precursor cells called perinatal oligodendrocyte type-2 astrocyte (O-2A) progenitor cells. In vitro, the O-2A precursor cell is bipotential, maintaining the capacity to differentiate into either oligodendrocytes or type-2 astrocytes (T2As) (Raff et al., 1983). Type-2 astrocytes are thought not to occur in vivo under normal conditions (Lemsky, 1997); thus it is generally assumed that astrocytomas arise from type-1 astrocytes or their progenitors. However, since some oligodendrogliomas progress to glioblastomas (Kocaeli et al., 2006) the implication would be that aberrations in oligodendrocytes could cause glioblastomas.

Astrocytomas are variable with respect to their morphology, therefore either they may arise from a similar population of cells which happen to sustain different initiating abnormalities, or there are already many heterogeneous types of astrocytes (Mayer-Proschel et al 1997, Rao 1999), astrocyte progenitors (Tole et al., 2000; Foley et al., 2000; Acampora et al., 2001; McCarthy et al., 2001; Martinez-Barbera et al., 2001; Hiroshi et al., 2002; Kiecker & Lumsden, 2004, 2005; Puelles et al., 2004, 2006; Shimogori & Grove, 2005; Takahasi & Liu, 2006; Emsley & Macklis, 2006; Nickerson et al., 2007), or stem cells (Sohur et al., 2006), which may account for some molecular differences in tumours (Shapiro & Shapiro, 1985; Bigner, 1981; Yung et al., 1982; Shapiro et al., 1991; Saxena et al., 1999; Camby et al., 2000; Nishizaki et al., 2000; Rich et al., 2005).

Figure 1-1 is a schematic diagram of neural stem cells (NSC) and their progeny in the developing forebrain. [The schema is reproduced from Merkle & Alvcarez-Buylla, 2006]. See explanation below. [Stri, striatum; VZ, ventricular zone]

The NSCs (shown in blue) lining the walls of the lateral ventricle change shape and produce different progeny as the brain develops. They begin as neuroepithelial cells and transform into radial glial cells, which mature into astrocyte-like cells. Typically, NSCs maintain contact with the ventricle into which they project a primary cilium. The potential of an individual stem cell in vivo is not known, however, the progeny shown in the schema are cell types that are generated by NSC. Stem cells produce progeny either directly or via an intermediate progenitor (some of which are shown here in green). Different types of progeny may be produced by different intermediate progenitors, although just one is shown here:

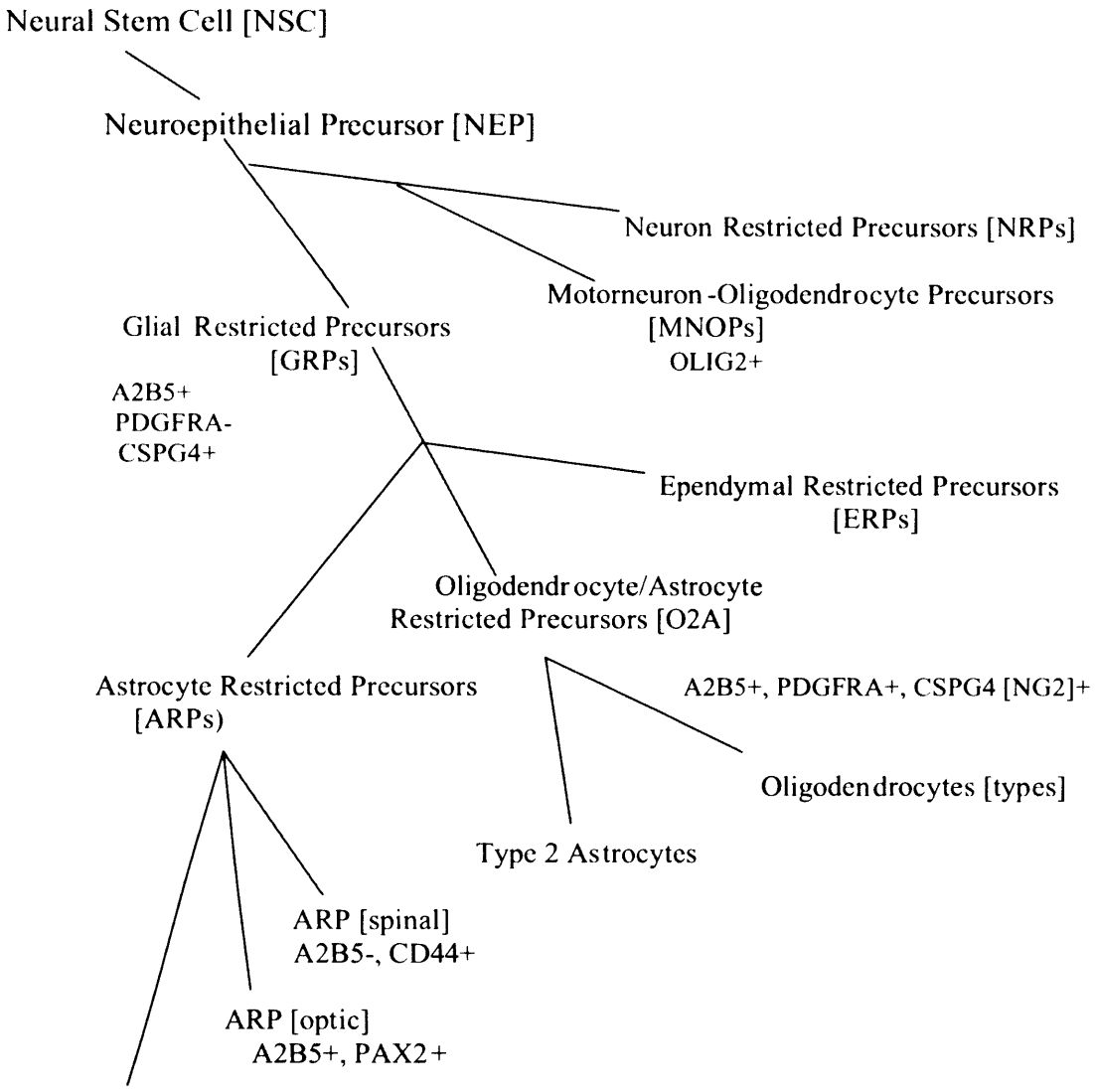
(a) At early developmental stages the CNS is a tubular structure. It is made up of neuroepithelial cells, which divide symmetrically at the ventricular surface to expand the stem cell pool. At this time, some early-born neurons such as Cajal-Retzius cells are produced.

(b) Neuroepithelial cells probably differentiate into embryonic radial glial cells, which divide to generate striatal neurons and oligodendrocytes either directly or via an intermediate progenitor in the subventricular zone (SVZ). The radial processes of radial glial cells support the migration of neuroblasts (shown in red).

(c) Radial glial cells persist in the neonatal brain, where they generate oligodendrocytes, olfactory bulb interneurons, and ependymal cells. They also generate astrocytes, some of which remain stem cells in the adult.

(d) In the adult brain, neurogenic astrocytes often retain a radial process and contact both the ventricle and the basal lamina of blood vessels. They generate oligodendrocytes and olfactory bulb interneurons.

Figure 1-2



1. ARP Cerebral: Probably subgroups exi st, based on anatomical/functional networks

   For example: Radial glia: NRG1+, ERBB2+, ERBB4+, BLBP+

   White Matter Precursor Cells (WMPCs): A2B5-

2. ARP Cerebellar Location

   E.g., Bergmann g lia: NRG1+, ERBB4+, ERBB2-, BLBP-

3. ARP of oth er brain parts: Mid-brain, Pons, etc., E.g., Polyadenocytes: CSPG4+, PDGFRA+

Figure 1-2 is a schematic representation of neural cell lineages: a model of established cells of origin of astrocytes and other known gli al cell types. [Modified from Mayer-Proscel et al., 1997; Richardson et al., 1997; Rao et al., 1999; Merkle & Alvarez-Buylla , 2006].

1-2 Cellular composition and locations of intracranial gliomas

Gliomas, of which astrocytomas and oligodendrogliomas are the most prevalent, develop from glial cells. It is not certain what proportion of the various glial tumours are histologically homogeneous, however, a glioma with more than one cell type is classified as mixed only if the most prevalent cell type is estimated to occur in <70% of the tumour (Cillekens et al., 2000). This definition raises interesting questions, particularly when viewed in light of the concept that it takes one aberrant cell, with the combination of mutations that confer a selective growth advantage, to trigger the formation of a clone that could result in a malignancy (Hanahan & Weinstein, 2000).

Approximately 94% of gliomas arise in intracranial sites (Brain Tumor Working Group: National Coordinating Council for Cancer Surveillance, USA, 1998), and the majority present in the supratentorial compartment in adults and the infratentorial in children.

1-3 Astrocytomas

1-3-1 Classification, Clinical features and genetic Characteristics of Astrocytic Gliomas

The WHO Classification puts astrocytic gliomas into 4 grades, I –IV, on the basis of histological appearances and biological behaviours (Kleihues & Cavenee, 2000; McCarthy et al., 2002). Grade 1 is composed of Pilocytic astrocytoma (ICD-O code 9421/1). They are predominantly benign, are well circumscribed and arise in familial /hereditary syndromes with astrocytoma association such as tuberous sclerosis and neurofibromatosis. Except for a small percentage, estimated as ~1.5%, pilocytic astrocytomas are surgically curable.

Grade II – IV astrocytomas are infiltrating and have therefore no defined border, which makes it impossible to cure them surgically. They invariably recur, with a tendency to progress to higher grades. Grade II astrocytomas (also known as diffuse astrocytomas) are of two recognised histological cell-types (Protoplasmic astrocytoma (ICD-O code 9410/3) and Fibrillary (Fibrous) astrocytomas (ICD-O code 9420/3). Grade III astrocytomas are also made up of two recognised histological cell-types: anaplastic astrocytomas (AA) (ICD-O code 9401/3) and Gemistocytic astrocytoma

(Gemistocytoma) (ICD-O code 9411/3). Grade IV is known as Glioblastoma, and is made up of astrocytic tumours constituting three separate ICD-O histological codes. The first of three ICD-O codes (9440/3) is made up by glioblastoma multiforme, spongioblastoma multiforme and glioblastoma NOS (not otherwise specified), the second ICD-O code (9441/3) is made up by giant cell glioblastoma and monstrocellular sarcoma) and the third (9442/3) by gliosarcoma and glioblastoma with sarcomatous component (McCarthy et al., 2002).

The prognosis for GBM is very poor. A EUROCARE study which looked at survival data from population-based registries of 18 European countries, altogether involving 6,513 adults diagnosed with GBM between 1990-1994, (followed-up until 1999), found relative survival rates of approximately 25% at one year, 5% at three years, and 3% at five years (Sant et al., 2003). The five-year relative survival decreased markedly with age from 11% in the younger (15-45 years) group to 2% in the group aged 75 years and over.

In the UK, the annual incidence of glioma is generally reported to be around 4 – 6 per 100,000 of the population (Brain Tumour Working Party, 2001). Thus, approximately 2,400 – 3,600 people develop glioma each year and most die within a year of the diagnosis (Maher et al., 2001; Sant et al., 2003; Ohgaki & Kleihues, 2005).

1-4 Long-term survivors

Long-term survival is currently defined as survival lasting 3 or more years from time of diagnosis (Scott et al., 1998; Burton et al., 2002). In spite of the overwhelmingly poor prognosis for high-grade gliomas, which is attributable mostly to astrocytomas, there are reports of GBM patients surviving for long periods. The Brain Tumour Study Party (2001) in a series of 467 patients found that 15-20% of the patients survived longer than 18 months. This survival was similar to that found by Lieberman and coworkers in a 1982 report of a study involving fifty-seven patients with malignant astrocytoma. Lieberman's group found a two-year survival of 14% (8 out of 57). As already reported, a more recent and larger study by ENCR (Sant et al., 2003) reported relative survival for patients with glioblastomas at 3 and 5 years, of 5% and 3%, respectively.

1-5 Risk factors

An increase in the incidence of brain tumours has been established definitively in association with advancing age; exposure to high doses of ionizing radiation, and in association with astrocytoma susceptibility- or familial/hereditary syndromes. Several epidemiological studies in addition to confirming the role of established risk factors as mentioned above, recently pointed to an association of glioma/astrocytoma with perinatal and late onset infections with the simian viruses (SV40 virus; Carbone et al., 2003), and high intake of proteins (Kaplan et al., 1997). SV40 is a known tumour virus however, the association with protein possibly implicated N-nitroso compounds, which are commonly associated with cured meats. The association with proteins is interesting since other studies such as that by Preston-Martin & Mark (1991), which have previously investigated the possible role of high intake of N-nitroso compounds in brain tumours were unable to find evidence specifically implicating N-nitroso compounds as risk factor for brain tumours.

1-5-1 Ethnic differences

The role of interethnic differences in the development of astrocytomas has yet to be ascertained. Interethnic genetic differences are believed not to play a significant part in brain tumours (Ohgaki & Kleihues, 2005; Miller et al., 2005), however some studies suggest that ethnic differences may indeed be important (Chen et al., 2001; Wang et al., 2004; Wiencke et al., 2005; Jemal et al., 2005). In 2001, Chen et al., reported findings of a population based study conducted in California, which investigated the prevalence and types of alterations in the p53 pathway genes in brain tumours. The study suggested that brain cancer incidence rates varied with race and ethnicity as well as gender. For example, Chen and colleagues found that non-Hispanic whites had brain tumour incidence rates 60–100% higher than in blacks, 10–90% higher than in Hispanics, and 40–45% higher than in Chinese, Japanese, or Filipino ethnicities. Within ethnic groups, rates were 7%, 39%, 66%, 71%, and 73 percent higher in men than women of Chinese, white non-Hispanic, black, Hispanic, and Filipino ethnicities, respectively.

1-5-2 Age

The incidence of brain tumours is highly correlated with age (Batchelor et al., 2004), there being, in general, an increase from around 2 per 100,000 for population group aged less than 20 years, to 20 per 100,000 for those aged 70 years (Surveillance, Epidemiology and End Results (SEER) in the U.S.A for the period 1975-1995 (Legler et al., 1999). These incidence rates are considered similar in most industrialised countries. The peak incidence in childhood, around ages 5-14 years (Legler et al. 1999), is followed by a marked increase after age 45, which reaches a high plateau around 65-75 years. Incidence is then reported to gradually decline to a sharp drop after the age of 80 years (Shugg et al., 1994), although this claim is disputed (Kleihues & Cavenee, 2000).

1-5-3 Gender

Regarding gender distribution, there is some uncertainty on the actual figures, however males are generally reported more affected than females. The prevalence is reported to vary from the lower figure of 6.4 per 100,000 population for males and 4.6 per 100,000 population for females (Shugg et al., 1994) to as high as 13.55 per 100,000 population compared to 12.25 per 100,000 population for males and females respectively (Ohgaki & Kleihues, 2005).

1-5-4 Environmental factors

Among several environmental factors implicated in human cancer in general, radiation has been convincingly implicated in the aetiology of gliomas. Therapeutic and prophylactic irradiation of the CNS of children with acute lymphocytic leukaemia (ALL) and pituitary adenomas is associated with increased risk of developing gliomas (Brat et al., 1999). Based on this established link with brain cancers, it is suggested that the age-related increase in brain tumour may arise out of accumulated toxicity or that it could be the consequence of prolonged latency (Lewis, 2005).

1-5-4-1 Exposure to electromagnetic fields

There is currently much concern about the effects of mobile phones, high-frequency radio transmitters and probable consequences of living near high power electric cables. In particular, considerable interest has focused on whether the use of mobile phones is associated with an increased risk of gliomas and other brain tumours, even though little is known about potential mechanisms (Sienkiewicz & Kowalczuk, 2005). The Advisory Group on non-ionizing Radiation (www.hpa.org.uk/radiation/), accessed in May 2007, suggest that the energy of the radiofrequency fields emitted by mobile phones is insufficient to cause malignant transformation through direct damage to DNA. Most published epidemiological studies on mobile phone use and gliomas have not generally reported any increased risk either overall, or with long term use (Muscat et al., 2000; Inskip et al., 2001; Lonn et al., 2005; Christensen et al., 2005). However, some isolated studies have found positive associations between high-grade astrocytoma (glioma) and phone use ipsilateral to the side of the tumour (Hardel et al., 2003), brain tumours and phone use in rural areas (Auvinen et al., 2002), and use of analogue mobile phones (Auvinen et al., 2003, Hardel et al., 2003). A large population based case-control study of 966 patients with glioma in the United Kingdom, part of the Interphone project started in 1999 (Cardis & Kilkenny), as part of an international collaboration of 13 countries investigating mobile phone use and the risk of intracranial tumours, reported in 2006 that use of a mobile phone, either in the short or medium term, is not associated with an increased risk of glioma. The authors of the report further concluded that the complementary positive and negative risks associated with ipsilateral and contralateral use of the phone in relation to the side of the tumour might be due to recall bias (Hepworth et al., 2006).

1-5-5 Exposure to viral infections

Three recent epidemiological studies (Fear et al. 2001; Dickinson et al 2002; McNally et al., 2004) investigated the role of prenatal and neonatal factors in the aetiology of CNS tumours in the UK. They all found an increased risk of brain and spinal tumours, notably astrocytomas and ependymomas among children exposed soon after birth to higher levels of community infections in particular measles and influenza.

1-5-5-1 SV40

There are many reports of detection of the simian virus 40 (SV40) DNA, and proteins, in human malignancies including brain cancers (Kouhata et al., 2001; Engel et al., 2005). SV40 is a known tumour virus and induces malignancies in laboratory animals (such as hamsters) that are identical to those in which its DNA is associated in humans (Butel & Lednicky, 2000). Known exposure to the SV40 virus has recently been linked to cancer development in a laboratory researcher who had a probable direct exposure, and subsequently developed a tumour positive for viral DNA sequences indistinguishable from those of the laboratory source (Arrington et al, 2003). This evidence strongly suggests a causal link between exposure to SV40 and development of brain tumours in humans

1-5-5-2 HCMV

HCMV is a beta-herpes virus that persistently infects 50-90% of the adult human population (Fish et al., 1996). It is trophic for glial cells (Fritschy et al. 1996) and is reportedly re-activated by inflammatory (Fritschy et al., 1996) and immunosuppressive (Lokensgard et al., 2002; Andre, 2005) conditions in the host. Its gene products can deregulate cellular pathways involved in regulating the cell cycle (Salvant et al. 1998; McElroy et al., 2000), apoptosis (Murphy et al., 2000), angiogenesis (Cinatl et al. 1999; Hoever et al., 2005), cues for cellular invasion (Scholz et al. 1999), and host immune response (Ross et al., 1987; Cinatl et al., 2005). Thus, it is probable that HCMV is directly implicated in the pathogenesis of several human malignancies (Shen et al. 1997), including glioma (Cobbs et al. 2002).

1-5-6 Probable aetiologic role of dietary/nutritional factors

N-nitroso compounds are implicated in carcinogenesis of the urogenital systems, and it appears that they may be involved in cancers of several other organs/systems (Okazaki et al., 2003). However, N-nitroso compounds and their precursors, nitrites and nitrates, have only been hypothesized as risk factors in brain tumours. Established inhibitors of N-nitroso compounds (e.g., vitamins C and E) are known anti cancer agents (Bunin et al., 1994). Several epidemiological studies have reported an association between

consumption of cured meats (a known source of N-nitroso compounds like nitrosamines) and cancers of the CNS in children (Dietrich et al., 2005; Kaplan et al., 1997).

1-5-7 Exposure to chemical carcinogens

Epidemiological studies have associated certain occupations with increased risks of brain tumour. In particular, risk has been associated with occupations involving exposure to formaldehyde (Krishnan & Levy, 1994; Andersen, 2003), rubber and plastics- (polyvinyl chlorides (PVCs), or polyaromatic hydrocarbons (PAHs) industries (Zheng et al., 2001). Other studies have reported increased risk among employees in heavy metal industries such as those with exposure to mercury, arsenic and lead (Navas-Acien et al., 2002). However, it appears that most of these studies have not been confirmed by independent studies in other populations, and no environmental carcinogen has been identified that could be unequivocally linked to brain tumour development in adults (Ohgaki & Kleihues, 2005). However, an international population-based case-control study conducted in 7 countries found an increased risk of glioma and other brain tumours in children born to parents employed in agricultural and textile industries (Cordier et al., 2001). Large population-based case-control studies have been carried out in seven countries as part of the IARC SEARCH program. Several of these studies suggest that parental occupation may influence the development of pediatric gliomas. http://monographs.iarc.fr/monoeval/crthall.html). The IARC monograph identifies 66 chemicals classified as carcinogenic to humans, 66 in Group 2A, that are categorized as probably carcinogenic, and 240 in Group 2B which are considered possibly carcinogenic to humans. In particular, the IARC monogram has identified exposure to nine chemicals with 'possible' or 'weak associations' with CNS tumours in humans. These are beryllium, epichlorohydrin, chlordane/heptachlor, methylthiouracil, thiouracil, propylthiouracil, lead, diisopropyl sulfate, and dichloromethane. The IARC monograph also contains various other chemicals that apparently increased the incidence of brain tumours (including gliomas) in experimental animals without concomitant evidence in humans. These included aflatoxin B1, diethyl sulfate, acrylamide, ethylnitrosourea, methylnitrosourea, procarbazine hydrochloride,

methyl methanesulfonate, dimethyl sulfate, glycidol, dacarbazine, 1,3-propane sulfone, and acrylonitrile.

1-5-7-1 Exposure to tobacco smoke

Long-term exposure to tobacco smoke is known to play a significant role in the aetiology of many solid tumours although an association with gliomas has not been demonstrated previously (Zheng et al. 2001; Peters et al. 2001). A recent clinical study of Efird et al., (2004), which investigated the association of tobacco smoking and other lifestyle behaviours and malignant primary adult-onset glioma (MPAG), reported increased risk among female smokers but not males. This study is significant in light of its unique design: it recruited a cohort of 133,811 adults aged at least 25 years, with no prior history of benign or malignant brain tumours, who were enrolled at the Medical Care Program of Northern California between 1977 and 1985. The average follow-up to the development of glioma was 21 years.

1-5-8 Genetic aetiology of cancer

Based on evidence gathered from observations going back over two centuries cancer was, until 2-3 decades back, viewed largely as an environmental problem. The observation by Percival Pott in the 19th century (Hall, 1998) that soot was a risk factor for scrotal cancer among chimney sweeps, and of a high incidence of cancers in populations of immigrants (Kolonel et al., 2004; Marchand & Kolonel, 1992), partly contributed to this view. Immigrants, for example, were observed to manifest altered risk of developing cancer when compared with the indigenous ethnic population. Advances in genetics in the past 15-25 years have provided supplementary evidence, which ultimately implicates molecular and genetic factors in causation of malignancies. At the cytogenetic and molecular levels, it is now accepted that a cancerous cell is likely to have sustained multiple genetic aberrations (Hanahan & Weinberg, 2000). These may involve copy-number or structural changes, and that other genes may be differentially expressed (Pillai et al., 2004). Altered genetic expression is usually the result of aberrations such as point mutations, translocations, genomic deletions, insertions and / or epigenetic modifications (Hulsebos et al., 2004). These aberrations vary between

tumour types, within the same type of tumour and grade in different people, and in different tumour grades in the same patient. Significantly, it is also known that there are intratumoural phenotypic differences, which are reflected in the histological appearances and malignancy grades in the same patient (Paulus & Peiffer, 1989; Coons & Johnson, 1993; Coons et al., 1997).

1-6 Initiation of the cancer process

Cancers arise from a complex multi-step process, resulting from accumulation of genetic aberrations, which confer a susceptible cell with properties that enable it to escape normal growth regulatory mechanisms. Once unregulated growth has been initiated, successive advantageous mutations then accumulate and promote clonal expansion of a malignant cell. Accumulation of mutations is vital for the acquisition of a genotype that allows the expression of characteristics typical of malignancy such as invasion of adjacent tissue, breaching the limiting membranes to cause metastases, and induction of malignant angiogenesis (Hanahan & Weinberg, 2000). Typically, it is estimated that the duration it takes from the time a cell sustains initial genetic damage to the appearance of manifest malignancy can be as long as several decades (Boehm & Hahn, 2005). Underlining the complexity of acquiring a malignant phenotype are thought to be essentially six transformations, 1) Self-sufficiency in growth signals, 2) Insensitivity to antigrowth signals, 3) Evading of apoptosis, 4) Unlimited replicative potential, 5) Sustained angiogenesis, and 6) Tissue invasion and metastasis (Hanahan & Weinberg, 2000; Gatenby & Vincent, 2002; Cristini et al., 2005). These genomic changes affect oncogenes, TSGs and DNA repair genes (Dunlop et al., 1997). Activated oncogenes are capable of inducing and maintaining neoplastic cell proliferation and tissue growth (Collins, 1995), whereas TSGs are negative regulators of growth (Knudson, 2001; Iwasa et al., 2005). DNA repair genes (Bartek & Lukas, 2007) maintain the integrity of the genome; following inactivation, there is increased genetic instability (Duesberg et al., 1998; Bartek & Lukas, 2007), which promotes tumour formation and growth (Yarosh et al., 2005).

1-6-1 Genetic aberrations: Tumour suppressor genes and Oncogenes

Although cancer genes are broadly classified as either TSGs or as oncogenes, by virtue of their specific roles/functions TSGs can be considered as concerned either with 1) maintaining the integrity of the genome, which includes control of entry into the cell cycle, 2) DNA repair, 3) mediating apoptosis, and 4) interaction/communication with the surrounding stromal tissues. TSGs are therefore further subdivided into roughly three categories, respectively, gatekeepers, caretakers, and landscapers (Kinzler & Vogelstein, 1998). Oncogenes, on the other hand, essentially promote growth as well as inhibiting differentiation and apoptosis. Uncontrolled expression of oncogenes and downregulation (or the complete inactivation) of TSGs leads to uncontrolled cell multiplication. In almost all cancers, abnormalities occur simultaneously in regulation of genes belonging to both classes.

1-6-2 Mechanisms of inactivation of tumour suppressor genes (TSGs).

TSGs are defined as the genes inactivated in conformity to Knudson's '2-hit' model; that is, both alleles of a gene are inactivated by genetic alterations such as chromosomal deletion and loss-of-function mutations. Typically, mutation or deletion is recessive at the cellular level since both alleles generally need to be inactivated to cause a tumourigenic effect (Knudson, 1971). In solid tumours such as astrocytomas, the first hit typically involves a small alteration while the second characteristically involves loss of large chromosome segments incorporating loci of many adjacent genes. Fully developed tumours may contain a heterogeneous collection of cells with so many chromosomal changes that it is difficult to extract clean patterns of loss specific to one small chromosome region using conventional LOH analysis. Because of this fact, the search for candidate TSGs using the traditional method of LOH analyses has quite often yielded disappointing results (Strachan & Read, 2007) considering that it involves looking at large areas of chromosomal changes, which could contain other probable candidate genes.

Once a candidate TSG has been identified, for example by LOH analyses or array CGH, further studies would follow to establish the expression profiles and functional relationship of the gene of interest. Gene expression studies, involving targeted

inactivation/deletion of candidate TSG might demonstrate that loss of function allows tumours to grow.

Figure 1-3a & b

Fig. 1-3 (a) (above) illustrates mechanisms of loss of wild-type allele in TSGs - Taking the example of retinoblastoma. (A) Loss of a whole chromosome by mitotic nondisjunction. (B) Loss followed by reduplication to give (in this case) three copies of the Rb chromosome. (C) Mitotic recombination proximal to the Rb locus (C_1), followed by segregation of both Rb-bearing chromosomes into one daughter cell (C_2). (D) Deletion of the wild-type allele. (E) Pathogenic point mutation of the wild-type allele. Red mark on chromosomes represented the RB1 locus on human CHR 13q.
(b) Shows results of typing normal (N) and tumour (T) DNA for the two markers A and B located as shown. (The figure is taken from Strachan & Read; Eds. Human Molecular Genetics 3, Chapter 17: Cancer genetics, pg. 495).

1-6-3 DNA methylation in astrocytoma

More recently it has become clear that methylation-related 'gene silencing' of the promoter region of many genes, or even silencing of vital transactivation domains away from gene promoter regions, acts as an alternative mechanism for aberrant TSG gene expression in solid tumours including astrocytomas (Uhlmann et al., 2003). Promoter hypermethylation and chromatin-structural-changes e.g., histone tail modifications (such as deactivation), cause transcriptional silence, and are important contributors to tumourigenesis (Costello et al., 2000; Nakamura et al., 2001; Jones & Baylin, 2002). DNA methylation is mediated by a family of DNA-methyltransferases including DNMT1, DNMT3A, and DNMT3B, which regulate a potent, heritable gene silencing system that is critical for normal embryonic development. This system is thought to be deregulated in nearly all tumor cells (Jones & Baylin, 2002). DNA methylation changes are thought to occur earlier and more frequently than individual genetic changes (Belinsky, 2004). Studies on the role of epigenetic silencing in glioma have been carried out using either a candidate gene approach and/or by a genome-wide screening method. For example, the former revealed that genes, such as TIMP3 (Lindsey et al., 2004; Martinez et al., 2007), whose gene product inhibits the activity of matrix metalloproteinases, and epithelial membrane protein (EMP3), a myelin-related gene believed to be involved in cell proliferation and cell-cell interactions, and are located within the commonly deleted 19q13.3 region, are frequently targeted for DNA methylation-mediated silencing in glioma (Alaminos et al., 2005). A genome-wide screen for aberrant DNA methylation events in glioma using restriction landmark genomic scanning revealed that 1,500 CpG islands may be subject to aberrant hypermethylation in low-grade gliomas, suggesting that epigenetic alterations are widespread and likely contribute significantly to gliomagenesis (Costello et al., 2000).

Epigenetic silencing of the MGMT gene, which is involved in the repair of DNA damage due to alkylation of the O6 position of guanine, is also frequent in glioma (Esteller, 1999). Silencing of MGMT by DNA methylation is associated with longer survival of newly diagnosed glioblastoma patients and is also an indicator of increased survival in patients treated with radiation plus the alkylating agent temozolomide (Hegi et al., 2005). However, the impact of MGMT methylation is much less clear and

controversial in the other treatment modalities including radiotherapy (RT) plus nitrosourea-based adjuvant chemotherapy, RT alone, and chemotherapy alone (Silber et al., 1999; Esteller et al., 2000; Paz et al., 2004). It is also not clear whether MGMT is by itself a prognostic factor or only a predictor of response to treatment. It has been suggested that in untreated low-grade gliomas MGMT was associated with shortened progression free survival (PFS) (Komine et al., 2003), and is due to a higher rate of GC to AT transitions targeting critical genes such as p53 (Watanabe et al., 2003, 2005), as the 06-alkylguanine may preferentially misrepair with thymidine rather than cytosine during DNA replication.

1-6-4 Mechanisms of oncogene activation

Unlike the classic TSGs, oncogenes require only one allele to be abnormal in order to exert their tumourigenic effect; thus they act in a dominant fashion (Hemminki & Li, 2004). Typically, oncogene activation occurs in one of 4 ways. 1) Generation of multiple gene copies (called amplification) (Kuttler & Mai, 2006) - sometimes in the form of small extra chromosomes, sometimes as duplications within a chromosome - often leading to overexpression. 2) A mutation may occur that alters the function of the protein encoded by the gene (Nenutil et al., 2005). For example, the three human RAS genes (KRAS, HRAS, NRAS) encode small GTPases, which activate an intracellular signaling cascade (RAS->RAF->MEK->MAPK) that leads to changes in gene expression. Gain of function mutations produce a hyperactive version of the GTPase that triggers excessive expression of the target genes. 3) A Chromosome rearrangement, usually a translocation (Truong & Ben-David, 2000), can up-regulate expression of an oncogene by moving it into a transcriptionally highly active region of chromatin where it comes under the influence of a strong regulatory promoter (Brake et al., 1998; 2002), leading to an inappropriately increased expression. A classic case is Burkitt's lymphoma in which the balanced reciprocal translocation - t(8;14)(q24;q32) – moves the MYC oncogene from CHR 8 to the neigbourhood of the IGH (immunoglobulin heavy) chain gene on CHR 14 and leading to overexpression of MYC by the cells/tissues (in the case of Burkitt's lymphoma, lymphocytes) with the translocation. 4) Finally, chromosome rearrangements can bring together exons of two distant genes to make a chimeric gene (Qian et al., 2005) usually resulting from a chromosome

translocation (Chernova et al., 2001), for example the BCR-ABL in CML, though it can sometimes follow an inversion or a deletion (Chernova et al., 2001).

1-7 Astrocytoma susceptibility [familial/hereditary] syndromes

A number of glioma/astrocytoma susceptibility syndromes have been described and are estimated to account for approximately 5-7% of sporadic astrocytomas (Acqui et al., 1989; Kleihues & Cavenee, 2000). The genes that are implicated in familial syndromes are 1) those for neurofibromatosis – NF1 (17q11.2), NF2 (22q12.2) (Evans et al., 2000; Gutmann DH, 2001). 2) Tuberous sclerosis – TSC1 (9q34), TSC2 (16p13.3) (Boesel et al., 1979; Arbiser et al., 2002). 3) Melanoma/astrocytoma syndrome (CDKN2AandCDKN2B; 9p21) (Kaufman et al. 1993; Randerson-Moor et al. 2001). 4) Nevoid basal cell syndrome (NBCCS) (or Gorlin syndrome - PTCH; 9q22.3) (Evans et al., 1991). 5) Hereditary nonpolyposis colorectal cancer type 1 (HNPCC1) MSH2; 2p22-p21)) (Okamoto et al., 2004; Vasen et al., 1996). 6) Hereditary nonpolyposis colorectal cancer type 2 (HNPCC2; 3p21.3) (Buerstedde et al., 1995). 7) Cowden syndrome, (PTEN; 10q23.3) (Zhang et al., 2000). 8) Turcot's syndrome, or familial adenomatosis polyposis coli/glioma syndrome, (APC; 7q21) (Yong et al., 1995; Hamilton et al., 1995), and 9) Li-Fraumeni syndrome (TP53; 17p13) (Tachibana et al., 2000; Rieske et al., 2005). However familial clustering of gliomas has been observed in the absence of these tumour syndromes (Paunu et al. 2002). Apart from these established astrocytoma susceptibility syndromes, many instances of astrocytoma have been noted in association with other cancer susceptibility-syndromes, e.g., Fanconi anaemia and breast cancer (Offit et al., 2003), Wilms tumour (Rainov et al., 1995; McNally et al., 2005) or other hereditary-disease syndromes, for example, ataxia-telangiectasia (Miyagi et al., 1995; Liang et al., 1995).

1-7-1 Germline mutations in astrocytoma susceptibility syndromes

It has been observed that quite often genes that are responsible for familial cancer syndromes are also altered in corresponding sporadic tumours (Lau et al., 2000; Paunu et al., 2001). In some cases knowledge of germline alterations characterised in familial cancers has aided the identification of somatic alterations in sporadic tumours and vice

versa (Kamb et al., 1994; Marsh et al., 1998; Paunu et al., 2001). Several alterations thought to be of critical significance for the development of sporadic gliomas have been identified including alterations of TP53, PTEN, CDKN2A and CDKN2B, and CDK4. In addition, the functional relevance of some of the implicated genes has been elucidated, and provides insights of pathogenesis of some tumours. For example, the TP53, CDKN2A and CDKN2B, and CDK4 genes are now known to serve as critical cell cycle regulators (Gemma et al., 1996; Fang et al., 2006). PTEN is a dual lipid and protein phosphatase associated with cell growth and migration (Kamb, 1995; Furnari et al., 1998). Thus, although this study is principally on sporadic high malignancy grade astrocytomas, it is relevant to provide a brief review of germline alterations that have been observed in some of the most common syndromes associated with susceptibility to astrocytomas.

1-7-1-1 Melanoma astrocytoma syndrome

The melanoma-astrocytoma syndrome is characterised by a dual predisposition to melanoma and tumours of the nervous system, in particular astrocytomas (Randerson-Moor et al., 2001). Germline deletions around 9p21 which involve the CDKN2A exon 1β, thought to implicate the coding region for the P16 and P14ARF (alternative reading frame) genes (Stone et Al., 1995), have been reported in patients of melanoma/astrocytoma and their relatives (Kamb et al., 1994). These mutations alter the protein coded by the genes. Mutational analyses of the CDKN2A gene in small groups of healthy individuals in several countries have detected the prevalence of variants in frequencies ranging from 6-48% among the French (Soufir et al., 1998), USA (Fitzgerald et al., 1996), Australian (Walker et al., 1995), Dutch (Gruis et al., 1995), Italian (Ghiorzo et al., 1996), Swedish (Platz et al., 1997), British (Harland et al., 2000) and Polish (Lamperska et al., 2002; Debniak et al., 2004; and Debniak et al., 2005) populations. Since only a limited number of individuals have so far been analysed, the overall frequencies of the CDKN2A variants are not known. However there are currently 77 CDKN2A variants listed in the International Melanoma Mutation Database: https://biodesktop.uvm.edu/perl/p16. All 77 result in altered protein products and may account for rare familial forms of melanoma, thus are believed to be causative. Two common polymorphisms in the 3-prime untranslated region (3' UTR) of CDKN2A

involving transition (mutation) of nucleotide (Nt) 500 from c to g (Nt500c>g), and Nt540c>t, are implicated in modulating the risk. The former is rarely associated with simultaneous mutations in other pathway genes such as CDKN2B, CDKN2C, CDK4 and P53 (Kumar et al., 2001). The latter is thought to modulate disease progression (Straume et al., 2002) because it has been observed to be frequently hypermethylated in melanomas of lower malignancy. The Polish studies found another common variant in which there is an alanine to threonine substitution at codon 148 (A148T), and is estimated to be present in approximately 3% to 3.5% of their population. It occurs more commonly in relatives of melanoma patients compared to the general population (3% vs 1.8%; Aitken et al., 1999). However, large germline deletions at the CDKN2A and CDKN2B (P16INK4A) tumour suppressor region involving the p16, p15, and p14 genes have been found in some families featuring both gliomas and cutaneous melanomas (Petronzelli et al., 2001; Debniak et al., 2004). It has not been established which, if any, of the above variants are associated with this predisposition.

1-7-1-2 PTEN hamartoma tumour syndromes

The PTEN hamartoma tumour syndrome, also known as Cowden syndrome, is a wide spectrum of disorders arising from germline mutations of PTEN of which familial glioblastomas is one possibility. Many malignant and benign lesions make up the syndrome: allelic variants constitute separate though related syndromes such as macrocephaly, lipomatosis, haemangiomatosis, and pigmented macules of the glans penis seen in the variant syndrome called Bannayan-Riley-Ruvalcaba (Marsh et al., 1999). Mucocutaneous lymphangiomatosis is the main feature of the variant known as Proteus syndrome (Biesecker et al., 1998), while dysplastic gangliocytoma of the cerebellum characterizes the Proteus-like and Lhermitte-Duclos disease (Zhou et al., 2000).

It has been suggested by Nelen and co-workers (1999) that the incidence of PTEN mutations, currently estimated to be 1 in 200,000, is at best a 'conservative' estimate. This view is based on the observation that penetrance is variable and age-related. Many others share this view, for example, Starink et al., (1986), Ohgaki et al., (2004). Thus,

for example, as a result of variable penetrance, in the study of Starink et al., (1999) most patients with PTEN hamartoma syndrome who developed breast cancers did so at around 38-46 years. However, with regards to brain tumours the lifetime risk for patients developing them in Cowdens syndrome is not known. Germline PTEN deletions and promoter mutations for Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome are estimated at 85–90% and 65%, respectively (Pilarski et al., 2004). Promoter mutations have only been noted in Cowden syndrome, while large deletions are observed only in Bannayan-Riley-Ruvalcaba syndrome and in patients with overlapping Cowden- and Bannayan-Riley-Ruvalcaba syndromes, but not Cowden syndrome (Zhou et al., 2003). Germline PTEN mutations are considered to be aetiologic for classic Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome, but have been found to be responsible for an increasing spectrum of clinical disorders as well as accounting for the majority of patients with adult onset Lhermitte-Duclos disease. It seems that glioblastomas have been observed in association only with the latter variant of the PTEN hamartoma syndromes (Kwon et al., 2001 & 2003). This is if interest, since Lhermitte-Duclos disease is noted to frequently manifest with atypical features, i.e., without signs typical of Cowden syndrome (Delatycki et al., 2003).

1-7-1-3 Li Fraumeni syndrome

Li-Fraumeni syndrome is an autosomal dominant disorder arising from germline mutations of TP53 and is characterized by multiple early-onset primary malignant tumours in childhood. These include brain tumours, sarcomas, adrenal carcinomas and leukaemia. However, in adults, cancers of the breast, soft tissue sarcomas, brain, bone, adrenal, leukaemia/lymphoma, stomach, colorectal, ovary, skin and cancers of many other sites are among the most frequent encountered (Olivier et al., 2003). The transcription factor P53 exerts its tumour suppressor function by activating target genes in response to stress signals such as those relayed following DNA damage, triggering the initiation of downstream effects like DNA repair, cell-cycle arrest, and / or apoptosis (Vogelstein et al., 2000). The p53 protein, composed of 393-amino acid (393-aa), has an amino-terminal transactivation domain, a core domain of approximately 200 amino acids (amino acids 96–292) that recognizes P53 DNA-binding sites (DBSs), and a carboxyl-terminal (C-terminal) tetramerisation domain. It is estimated that up to half

of all human cancers carry P53 gene mutations, of which many have been documented in large international databases (Beroud & Soussi, 2003). The International Agency for Research on Cancer (IARC) TP53 Mutation Database, www.iarc.fr/p53, for example, contains 264 mutations described in 261 families and 19,809 somatic p53 mutations (IARC web, accessed in June 2005) of which approximately 71% result in full-length protein with only one amino acid substitution within the p53 core domain (Olivier et al., 2003). In addition, p53 mutations are frequently found in solid tumours including astrocytomas, that are resistant to conventional therapies (Beroud & Soussi, 2003). Interestingly, 8 amino acid changes and 50 amino acid changes, respectively, account for 30% and 55% of all core domain mutants with a single amino acid change (Olivier et al., 2002).

1-7-1-4 Tuberous sclerosis

Tuberous sclerosis is characterized by development of tumour-like growths, called hamartomas in the skin and several other organs especially the brain and kidneys. It is associated with mental retardation and epilepsy. Hamartomata in the CNS are differentiated benign growths forming localised regions of disorganised cortical architecture comprising abnormally shaped neuronal cell body, dendrite processes, and spine density. Among CNS manifestations brain tumours, in particular subependymal giant cell astrocytomas (SEGA) and seizures (which occur in 80-90% of affected individuals) are a major cause of morbidity (Parry et al., 2000). Astrocytomas have also been described in association with TSC (Parry et al., 2000; Kwiatkowski et al., 2003). Germline mutations of two TSC genes, TSC1 at 9q34 encoding a 130kDa protein named hamartin (The TSC1 Consortium, 1997), and TSC2 (16p13.3) encoding tuberin, which is a 200kDa protein (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993), are transmitted in an autosomal dominant fashion. The prevalence is estimated to be 1 in 6,000-10,000 individuals, of which approximately two-thirds are sporadic. Among familial cases, linkage analysis suggests that about half of large families are linked to TSC1 and half to TSC2, while sporadic cases are more commonly associated with mutations involving the TSC2 gene (Kwiatkowski et al., 2003). The range of mutations, which have been described for TSC2 is wide and heterogeneous and includes large deletions/rearrangements as well as insertions, deletions, nonsense,

splicing and missense mutations. On the other hand, the vast majority of TSC1 mutations are small missense or deletions, nonsense or splicing mutations (Kwiatkowski et al., 2003).

In renal lesions the TSC1 and TSC2 genes act as tumour suppressors and show LOH in lesions, which appear to be monoclonal in origin (Murthy et al., 2000). In brain lesions, it is not quite so clear – there are publications, which suggest that haploinsufficiency may be the underlying mechanism in some brain lesions (Goorden et al., 2007; Wienecke et al., 2002). Studies of CNS abnormalities and tumour predisposition in the Eker rat, a naturally occurring rat model with known TSC2 mutations (Yeung et al., 1994; Kobayashi et al., 1995; Yeung RS, 2004), and studies of astrocyte-specific TSC1 and TSC2 knockout models (Uhlmann et al., 2002; Potter et al., 2001) have revealed some ways in which TSC1 and TSC2 genes might contribute to events in early/embryonic development (Yeung et al., 1994). The TSC genes exert influence on the G0/G1 – S transition (Soucek et al., 1997; Uhlmann et al., 2002) by regulating levels of the CDK inhibitor (CKI) p27 (Uhlmann et al., 2002; Khare et al., 2003). Deficient levels of TSC genes cause increase in cell cycle progression with corresponding increase in cell proliferation (Catania et al., 2001). In addition, the TSC genes appear to regulate cell size (Cooper, 2004). This function is typically attributed to genes involved in the insulin-signalling pathway such as insulin growth factor (IGF) molecules and their receptors PI3K, PKD1, AKT and TOR, and is normally regulated by PTEN (Potter et al., 2001; Gao & Pan, 2001). The mechanism by which TSCs are able to control the growth of cells has recently been linked to regulation of the molecular pathways involving mammalian target of rapamycin (mTOR), which is negatively regulated (inhibited) by the TSC1/TSC2 complex, acting via a downstream inducible GTPase target of TSC2 that is known as Rheb (Tee et al., 2003; Smith et al., 2005).

1-8 Model for the molecular/genetic basis of astrocytomas

Diffuse astrocytomas infiltrate throughout the brain making complete surgical removal virtually impossible (Frank et al., 1988; Guyotat et al., 2000) and, in addition, they are almost invariably resistant to radiation and chemotherapy (Lefrank & Kiss, 2005, 2006;

Jagannathan et al., 2006). It is apparent from clinical characteristics of grade IV astrocytomas that gliomas are heterogeneous. Grade IV astrocytomas are divided into primary and secondary GBMs: the former arising de novo whereas secondary GBMs develop progressively from a low-grade astrocytoma (Kleihues & Cavenee, 2000; Maher et al., 2001; Holland, 2001). Cytogenetic studies of lesions followed up by molecular studies have suggested developmental pathways of gliomagenesis. Genetic studies, particularly of secondary GBMs, indicate that there are distinct genetic pathways involved in initiation and progression of astrocytic neoplasms

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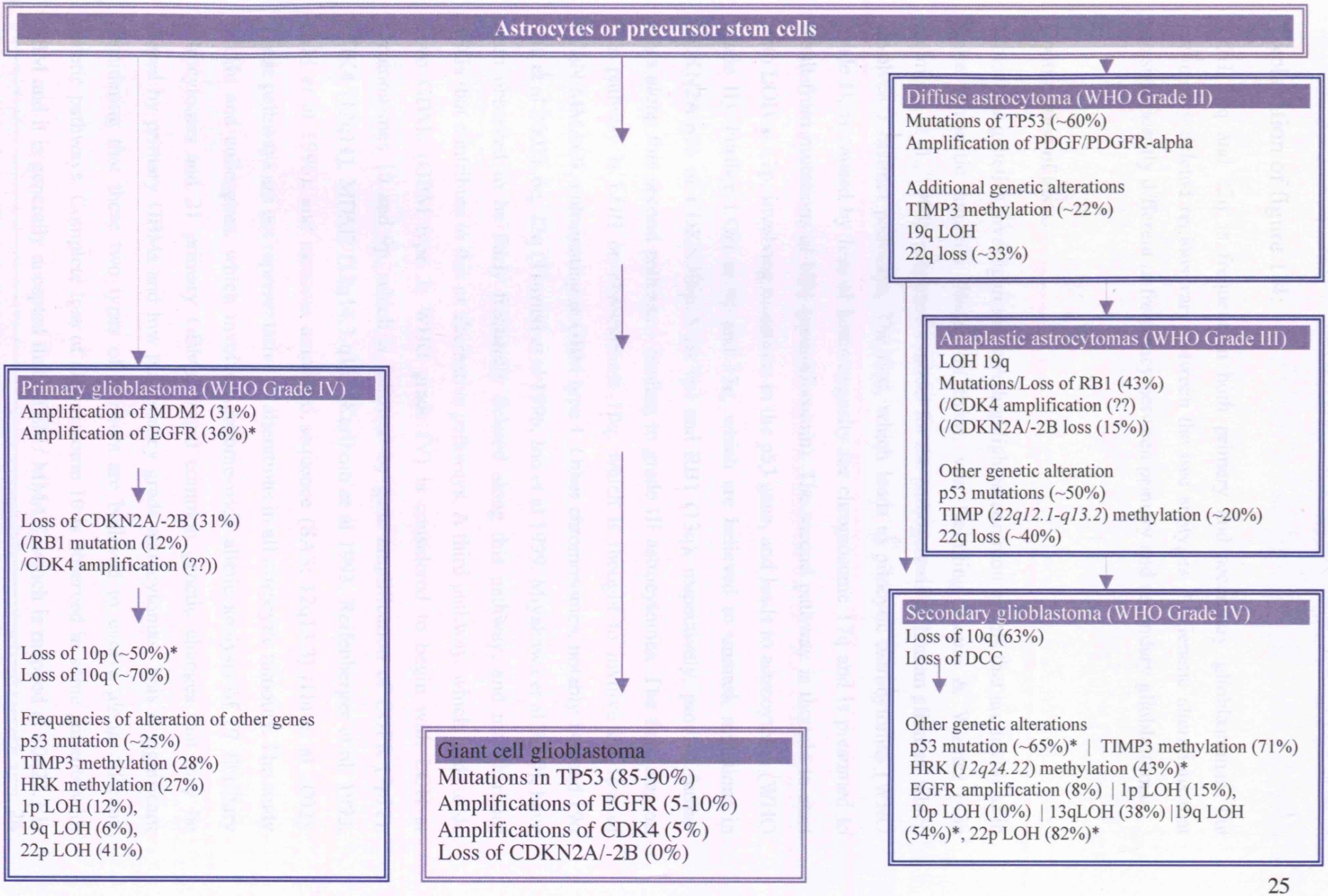
Fig 1-4: Model of genetic pathways to primary and secondary glioblastomas (adapted from von Deimling et al., 1997; Reifenberger et al., 1997; Hong-Xuang et al., 1998; Ohgaki et al., 2004; Ohgaki, 2005; Meyer-Puliz et al., 1997; Martinez et al., 2007



Explanation of figure 1-4:

LOH 10q and 22q is frequent in both primary and secondary glioblastomas, but commonly deleted regions vary between the two subtypes. *, Genetic alterations that are significantly different in frequency between primary and secondary glioblastomas.

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Molecular genetics investigations have highlighted common events that are involved in some astrocytic tumours. Based on these, von Deimling, Louis & Wiester (von Deimling et al., 1995), proposed a model for the pathogenesis of human gliomas, which involves 3 distinct pathways. The first, which leads to pilocytic astrocytomas (WHO grade I), is caused by loss of heterozygosity for chromosome 17q and is presumed to result from mutations of NF1 (neurofibromin). The second pathway is thought to start with LOH at 17p, involving mutations in the p53 gene, and leads to astrocytoma (WHO grade II). Further LOH at 9p and 13q, which are believed to unmask mutations in CDKN2A/p16 or CDKN2B/p15 (at 9p) and RB1 (13q), respectively, provide further steps along this second pathway - leading to grade III astrocytomas. The final step on this pathway is LOH on chromosome 10q, which is thought to involve the loss of PTEN/MMAC1, culminating in GBM type 1. Other chromosomes, notably 14q and 19q (Hu et al 2002), 6q, 22q (Tsuzuki et al 1996, Ino et al 1999, Miyakawa et al 2000), have been observed to be fairly frequently deleted along this pathway, and may harbour TSGs that contribute to this or alternative pathways. A third pathway, which leads to de novo GBMs (GBM type 2; WHO grade IV) is considered to begin with LOH at chromosomes 10 and 9p, which is followed by gene amplification of EGFR (7p12), CDK4 (12q14), MDM2 (12q14.3-q15) (Karlbom et al 1993, Reifenberger et al. 1993, Ueki et al 1996), and sarcoma amplified sequence (SAS; 12q13.3) (Hu et al 2002). These pathways are not representative of alterations in all astrocytic tumours. The study by Hu and colleagues, which involved genome-wide allelic analysis of 17 fibrillary astrocytomas and 21 primary GBMs, found common genetic changes that may be shared by primary GBMs and low malignancy grade astrocytomas. This is significant considering that these two types of tumours are believed to evolve along different genetic pathways. Complete loss of chromosome 10 is observed in some patients with GBM and it is generally accepted that PTEN / MMAC, which is mapped at 10q23.3 is

critical in transformation of AAs to GBMs (Kotelevets et al., 2001; Hu et al., 2002; Leuraud et al., 2003a). Partial losses involving at least 2 additional common regions of deletion (CRDs) on chromosome 10 are frequently observed at 10p14-p15.1 and 10q 25.1-qter (Hu et al 2002). As yet it is not clear what genes, in these locations, are implicated or if they have critical roles in transforming astrocytes. However Nishimoto et al. (2001) mapped a repressor of telomerase expression to a 2.7-cM region on 10p15.1. This candidate gene is a tumour suppressor and was found inactivated in high-grade gliomas (Leuraud et al. 2003b).

1-8-1-1 Progression pathways of astrocytoma development

Genetic pathways that are specifically disrupted in high-grade- but not in low-grade astrocytomas are considered to be involved in progression (Rickman et al., 2001; Markert et al., 2001). To maintain normal homeostasis cells have several mechanisms to regulate cell-cell progression, one of which takes place at the G1/S checkpoint (Castro et al., 2005). This checkpoint is believed to be primarily under the control of the RB genes (Fang et al., 2006). A hallmark of high-grade astrocytomas is high mitotic activity (Coons & Pearl, 1998; Colman et al., 2006) and is believed to reflect the frequency of aberrations involving components of the RB-CDK-CKI (cyclin-dependent kinase inhibitor) pathway (Xiao et al., 1995; Yin et al., 2002).

Figure 1-5: G1-S checkpoint

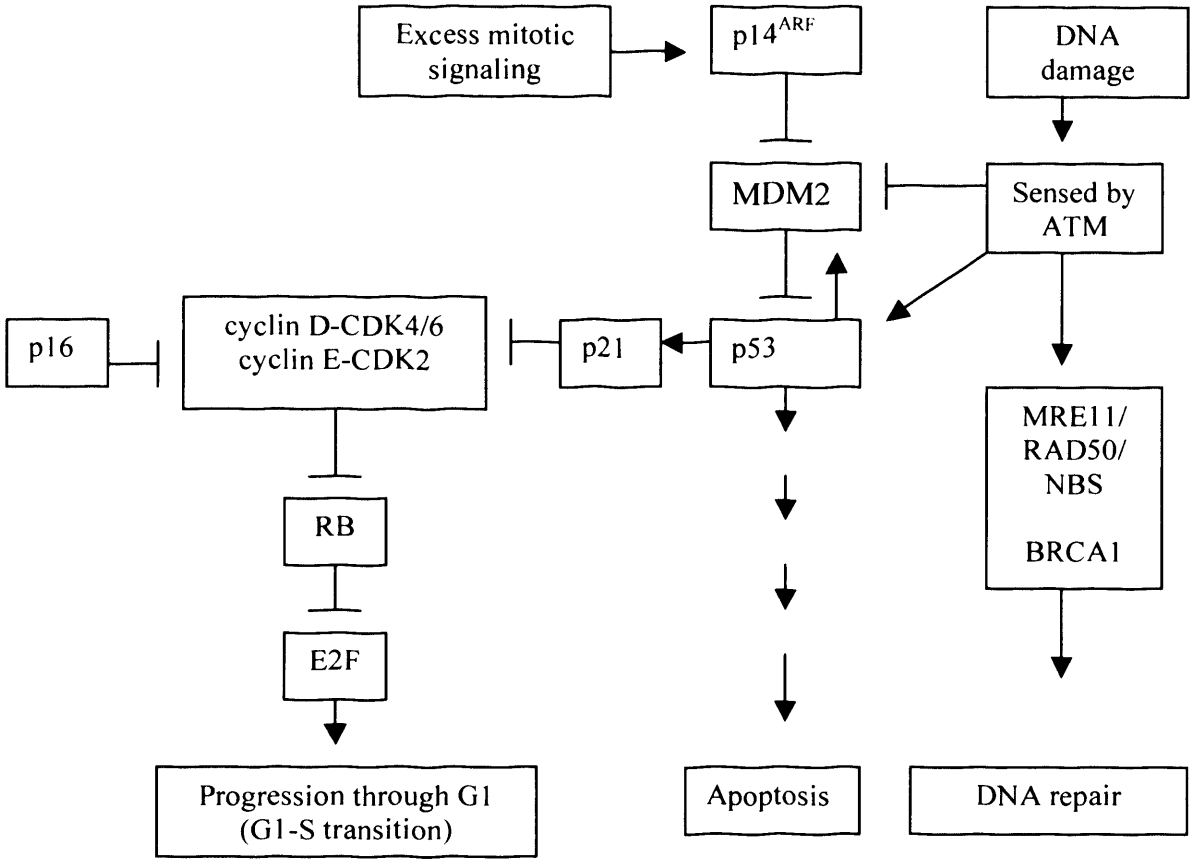


Figure 1-5 shows some of the controls and interactions governing G1-S progress.
⊣ Shows inhibitory action, → Shows a stimulatory action.

Loss of CDKN2A is reported to occur in 40-57% of GBMs (Ichimura et al., 1996), CDK4 amplification is found in 12-14% of GBMs (Ichimura et al., 1996; Burns et al., 1998), while loss of RB is identified in 14-33% of GBMs (Ichimura et al., 1996). These genetic alterations are found to be almost invariably mutually exclusive (Biernat et al., 1997; Wilkman & Kettunen, 2006) which is believed to imply that mutation of any component of the CDKN2A-CDK4-RB pathway can have equivalent effects (Nakayama et al., 1996). From a histological point of view this may well be, however recent findings from a glioma model seem to suggest that there could be important differences, particularly as regards the biological behaviours (Hesselager et al., 2003). Mutation of components of the RB pathway combined (INK4A/CDK4/RB) are detected in >80% of GBMs and in ~50% of AAs (Biernat et al., 1997). It is thought that the remaining 20% of GBMs that lack detectable INK4A/CDK4/RB mutations could

harbour mutations in other components of this pathway. Indeed, by way of substantiation, overexpression of CDK6 and cyclin D1 has been detected in a number of GBMs (Costello et al., 1997) while in one study downregulation of E2F1 and overexpression of cyclin E were observed in some high-grade gliomas (Walter et al., 2002). Data from mouse models support the idea that genes involved in RB-mediated cell-cycle progression checkpoint are important in progression but not initiation of astrocytoma (Sharpless et al., 2001). In their study on Ink4a-homozygous and Ink4a-heterozygous mice, Sharpless and colleagues, found that neither group of mice developed astrocytomas although they were considered cancer prone. Similarly, the study of Holland et al., (2000) on Rb -/- astrocyte cultures, and that of Merlo et al., (2003) on mouse models with overexpression of Cdk4, both groups failed to develop astrocytomas. However, simultaneous inactivation of Rb protein pathway in mice and overexpression of a truncated SV40 T antigen led to development of high-grade astrocytomas (Xiao et al., 2002).

1-8-2 Genetic alterations with probable initiating roles for astrocytoma

Cytogenetic profiles of astrocytomas of various tumour grades reveal alterations that are common to low-grade- and high-grade tumours, and others that are restricted to tumours of higher grades. This led to the hypothesis that mutations that are shared by tumours of low- and high grades may be involved in early phases of tumour formation (Vogelstein et al., 2000). So far, the TP53 TSG, the receptor tyrosine kinases (RTKs) and RAS are the leading candidates genes that are implicated in the initiation of astrocytomas (Paulus et al., 1996; Nozaki et al., 1999).

1-8-2-1 The Role of TP53 Tumour Suppressor Gene

TP53 is a transcription factor that regulates cell cycle progression and apoptosis in response to a variety of external insults, such as DNA damage and oncogenic mutations (Vogelstein et al., 2000). Germline mutations of the P53 gene cause the Li-Fraumeni Syndrome; a familial cancer syndrome that predisposed affected individuals to development of various brain tumours including astrocytomas (Malkin et al., 1990). Mutations of TP53 are found with equal frequency (estimated at >60%) in all grades of sporadic astrocytomas (Louis DN, et al., 1993; Ohgaki, et al., 1999). Since

astrocytomas, including the well-differentiated ones, exhibit a unique tendency to infiltrate surrounding tissues, a trait that is not common in other neoplasms until they have attained advanced stages (Kinzler & Vogelstein, 1996), the association of this tendency with the high frequency of mutations of the TP53 is tempting. The early loss of TP53 is also implicated in the ability of astrocytomas to evade apoptosis, thereby continuing to migrate and survive in a microenvironment that would normally not provide adequate growth support (Rich et al., 2005). However, in spite of the evidence from genetic studies pointing to an association between TP53 mutations and initiation of astrocytomas, studies of mouse models have not demonstrated a causal link. Instead the mouse models demonstrate that loss of Tp53 alone is insufficient to initiate astrocytoma formation (Donehower et al., 1992; Jacks et al., 1994), which implies that additional genetic or epigenetic events are required (Watanabe et al., 2003; Ichimura et al., 2000).

1-8-2-2 The Role of Receptor Tyrosine Kinase (RTK) and RAS

Normal cells require growth signals for survival and/or proliferation (Yu et al., 2002). Many growth signals are mediated by diffusible growth factors transmitted into the cell via transmembrane proteins with intrinsic RTK activity (Burdon et al., 1999). RTKs bind to growth factors resulting in receptor dimerisation, autophosphorylation and recruitment of adaptor proteins such as growth factor receptor bound protein 2 (GRB2) and sarcoma (Src) homology 2 domain containing (SHC), which interact with and activate various downstream effectors. One example of downstream effectors of RTK signaling is the Ras oncogene, which codes for a small GTP-binding protein (Seger & Krebs, 1995). Ras can activate at least three downstream cascades: 1) RAF- (mitogen activated, extracellular signal regulated kinase) MEK -MAPK (mitogen-activated protein kinase), 2) phosphatidylinositol 3-kinase (PI3K)-AKT, and 3) CDC2-RAC-RHO (Seger & Krebs, 1995; Hunter, 1998). The growth factor-RTK-RAS signalling cascade is one of the most frequently targeted genetic pathways in human cancers (Takahashi et al., 1992; Weis et al., 1999), which could suggest that activating mutations of this pathway render cancer cells independent of exogenous growth factors (Yamaguchi et al., 1994; Guha et al., 1995; Parsa & Holland, 2004). Genetic analysis of astrocytomas reveals elevated expression of several growth factors and their RTK

receptors, for example PDGFR, in all grades of astrocytoma (Hermanson et al., 1996), which could indicate that RAS-mediated signaling is involved in initiation of astrocytoma development (Parsa & Holland, 2004).

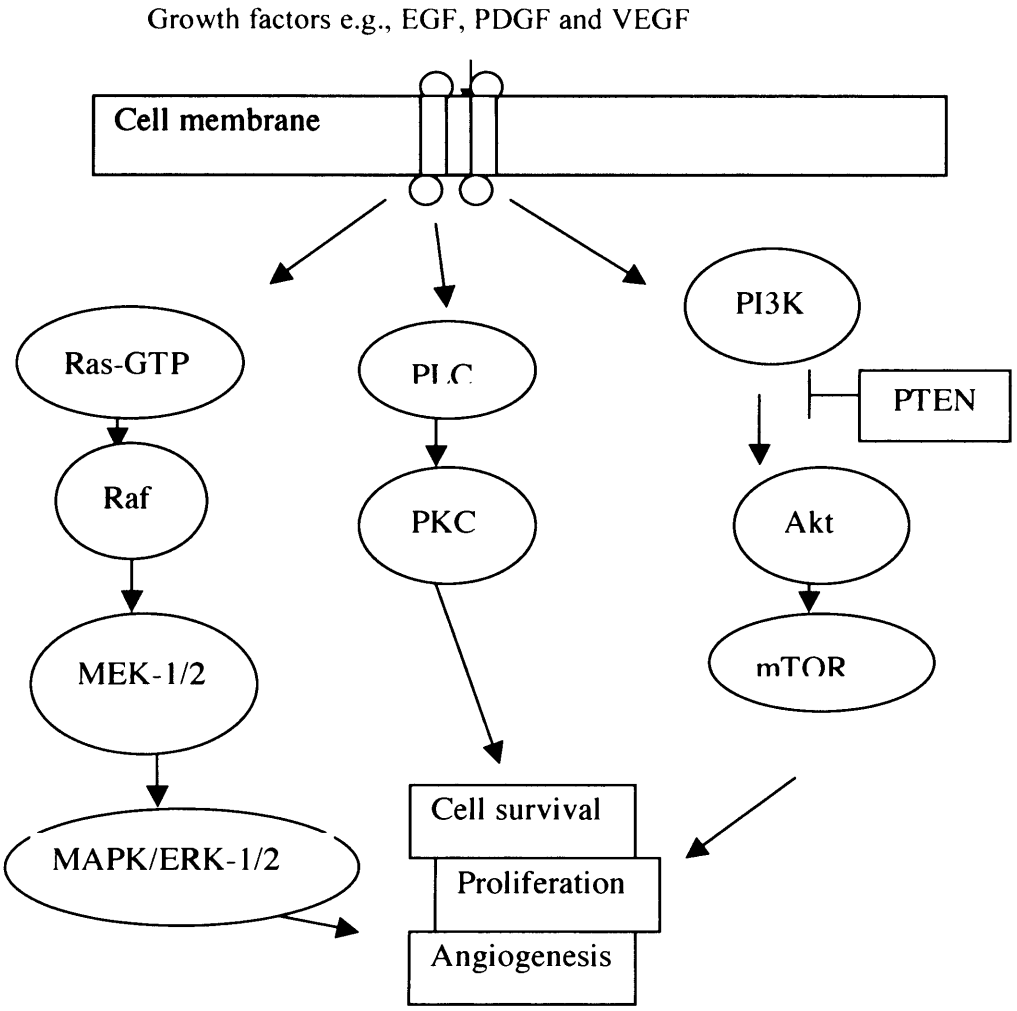


Figure 1-6 shows the growth factor signaling pathways

The tyrosine kinase activity of growth factors is stimulated after binding to the cognate growth factors, which results in the stimulation of multiple downstream signaling cascades. These pathways are important in various cellular functions, including cell survival, proliferation and angiogenesis. As a result, novel molecular therapies for high grade astrocytomas are being sought that target various factors in these pathways e.g., farnesyltransferase inhibitors RTK->Ras-GTP); Raf inhibitors (Raf); PKC-B2 inhibitors (PKC); RTK inhibitors (EGFR, PDGFR, VEGFR); mTOR inhibitors (mTOR).

EGF, epidermal growth factor; EGFR, EGF receptor; PDGF, platelet derived growth factor; PDGFR, PDGF receptor; MEK-1/2, mitogen-activated protein kinase-1/2; PLC, phospholipase C; PKC, protein kinase C; PI3K, phosphoinositol-3-kinase; mTOR, mammalian target of rapamycin; Akt, protein kinase B; PTEN, phosphatase and tensin homology deleted on chromosome 10. The figure is adapted from Brandsma & van den Bent, 2007.

PDGF and PDGFR in particular are often co-expressed in the same tumour cells, suggesting that astrocytoma cells establish an autocrine stimulatory loop (Nister et al., 1988; Hermanson et al., 1992; Rajasekhar & Holland, 2004), and could therefore be involved in mediating astrocytoma development (Nister et al., 1988; Vogelstein et al., 2000). This possibility is supported by experiments in mice in which Pdgf was expressed in the brains using a retrovirus vector (Uhrbom et al., 1998), resulting in development of astrocytomas in 40% of the mice. These tumours also expressed Pdgfr, thus supporting a model of autocrine stimulation (Hermanson et al., 1992; Uhrbom et al., 2002). However, these mice experiments also seem to suggest that co-expression of Pdgf/Pdgfr may be required for astrocytoma to develop only in certain situations, for example, astrocytoma models that have been developed by Pdgf overexpression are unable, so far, to develop low-grade astrocytomas (Uhrbom et al., 1998; Dai et al., 2001) as might be expected in the early stages of tumour development. Perhaps the explanation lies in the stage of development of these mouse models, which are typically neonates (Oumesmar et al., 1997). Taken together with data from mouse experiments, it appears that since astrocytomas occur mainly in adulthood in humans (Kleihues & Cavenee, 2000) either co-expression of Pdgf/Pdgfr is a requirement for tumour initiation only in adult animals, including humans, or it may be that adult astrocytes are the more susceptible target for Pdgf signalling (Zhu & Parada, 2002). An additional issue concerning the role of PDGF/PDGFR as probable mitogens for initiation of astrocytomas is that no mutations have been detected in either PDGF or its receptor in astrocytomas (Saxena et al., 1999; Dai et al., 2001; Hasselager et al., 2003). Moreover no defects of astrocyte development have been reported in Pdgfa- or Pdgfb-null mice (Fruttiger et al., 1999), both of which observations render uncertain the precise role of PDGF/PDGFR signalling in astrocyte in both initiating and/or promoting development of astrocytic tumours.

Furthermore, PDGF/PDGFR signaling is typically thought to be mitogenic (Saxena et al., 1999; Dai et al., 2001; Hasselager et al., 2003) which is also the primary basis for implicating these genes in astrocytoma oncogenesis. However, low-grade infiltrating astrocytomas typically have low mitotic activity despite being invasive (Johannessen & Torp, 2006). This would appear to suggest that mitogenic factors by themselves are not a critical requirement for development of low-grade astrocytomas (Hasselarger et al., 2003). If PDGF/PDGFR signalling is involved in astrocyte development it could possibly be involved in functions other than or in conjunction with proliferation: for example, a PDGF/PDGFR could be involved in regulating cellular migration as demonstrated for a Drosophila PDGFR homologue by Duchek et al. (2001).

RAS mutations are found in ~30% of human cancers in general (Rushworth et al., 2006), but, apparently, have not been detected in astrocytomas (Maltzman et al., 1997; Knobbe et al., 2004) although Sharma et al., (2005) have reported finding what they believe to be an oncogenetic mutation in one out of 21 (4.8%) pilocytic astrocytoma in their study. More commonly, RAS appears to be activated indirectly by upregulation of growth factor-RTK pathways (for example PDGF/PDGFR), through the loss of the NF1 gene (Cickowski et al., 2003) or via other RAS-pathway-activating mechanisms (Kaplan & Stephens, 1994; Nakamura, 1995). The NF1 gene encodes the protein neurofibromin, which shares homology with the RAS GTPase-activating protein (GAP) family (Oguzkan et al., 2006). RAS-GAPs catalyse conversion of activated RAS-GTP to RAS-GDP, thereby downregulating RAS activity (Oguzkan et al., 2006). Patients with neurofibromatosis type 1 are predisposed to developing astrocytomas (Cickowski et al., 2003), which are mostly believed to be of the pilocytic type (Kleihues and Cavenee, 2000). Homozygous deletion of the NF1 alleles has been demonstrated in these tumours (Tada et al., 2003). However, activation of RAS-mediated MAPK and PI3K cascades are found in both NF 1-mutant-associated and sporadic astrocytomas (Lau et al., 2000). Although no mutations of Ras have been observed in astrocytomas, direct evidence implicating Ras in astrocytoma oncogenesis was provided by a mouse transgenic model (Bajenaru et al., 2002) in which overexpression of oncogenic Ras in astrocytes led to the development of astrocytomas.

1-9 The pathogenesis & biological characteristics of primary GBMs

Although primary and secondary GBMs have similar histopathological characteristics and apparently clinical outcomes (Kleihues & Cavenee, 2000) the kinetics of tumour development in these two subgroups are strikingly different (Kleihues et al., 2000). Primary GBMs arise rapidly, typically in <3 months, and without clinical or histological evidence of pre-existing low-grade lesions (Kleihues & Cavenee, 2000; Maher et al., 2001). For this reason, it is difficult to distinguish between genetic alterations that contribute to their initiation and those associated with progression (Mao & Hamoudi, 2000; Weinberg, 2005). However, mutational analysis indicates that some of the genetic pathways that are disrupted in secondary GBMs are also altered in primary GBMs (Kleihues & Cavenee, 2000). A question thus arises regarding what mechanisms might explain the differences between the rapid- versus slow-progression in glial tumours (Kleihues & Cavenee, 2000). Certain genetic alterations have been observed that could separate primary from secondary GBMs.

1-9-1 INK4A/ARF/TP53 mutations

Mutations in INK4A, which are found in ~40% of GBMs, most of which are homozygous deletions (Labuhn et al., 2001; Houillier et al., 2006), are common in primary GBMs as compared to secondary GBMs, of which only ~4% have INK4A mutations (Biernat et al., 1997). TP53 mutations are more frequently detected in secondary GBMs (~60%) but are found in ~10% of primary GBMs (Lang et al., 1994). In these tumours, mutations of TP53 and INK4A are mutually exclusive (Fulci et al., 2000) and are believed to be due to a shift in the INK4A reading frame at the CDKN2A locus leading to a coding of the CDKN2B (P14ARF) gene in GBMs without INK4A mutations (Sherr et al., 2001; Bruggeman et al., 2005). ARF stabilizes p53 proteins through binding to MDM2, which normally targets p53 for ubiquitin-mediated degradation (Sherr et al., 2001). Thus, in secondary GBMs, TP53 pathway is directly mutated whereas in primary GBMs the p53 pathway is altered resulting either in loss of ARF or upregulation of MDM2. Homozygous deletion of the CDKN2A locus ablates both INK4A and ARF function (Labhun et al., 2001), simultaneously affecting the RB and p53 pathways (Labhun et al., 2001; Uhrbom et al., 2005). It has been suggested, for example, by Miao et al., (2001) and Weinberg, (2005) that homozygous inactivation of

the INK4A locus might explain why primary GBMs manifest so rapidly (Bruggeman et al., 2005). If this is the case, it could suggest that simultaneous loss of two key growth-regulatory pathways in a cell might expose it to more favourable conditions for development of cancer (Miao et al., 2001; Weinberg, 2005). This concept is supported by studies in mouse models of astrocytoma in which simultaneous disruption of Nf1 and Tp53 genes results in development of high-grade astrocytomas whereas a step-wise loss of the same genes does not (Reilly et al., 2001).

1-9-2 EGF/EGFR

Amplification of EGFR is found in ~40% of primary GBMs but rarely in secondary GBMs (Lang et al., 1994). EGFR amplification is associated with mutations in the genes for INK4A and is mutually exclusive with mutations of TP53 (Simmons et al., 2001). Most astrocytomas with EGFR amplifications (estimated to be ~77%) have additional genetic alterations (Frederick et al., 2000) most of which are intragenic rearrangements that lead to truncated and constitutively active EGFR (Sugawa et al., 1990; Frederick et al., 2000). Overexpression of truncated EGFR alleles confers a growth advantage and tumourigenic properties in glioma cell lines (Nishikawa et al., 1994). In vitro administration of dozes of Egfr to brains of neonatal mice, in amounts equivalent to overexpression, failed to induce tumour formation in normal mice while those with mutations of Ink4a-Arf developed brain tumours (Holland et al., 1998). This seems to provide proof that EGFR activation in the background of Ink4a-Arf deficiency in some ways corporate to cause development of astrocytic tumour (Holland et al., 1998).

1-9-3 PTEN

Loss of the 10q arm is the most common genetic alteration associated with GBMs (Fults et al., 1993; von Deimling et al., 1993), and several GBM-associated genetic loci, among them loss of PTEN (Li et al., 1997) have been identified (Fults et al., 1998). Alterations of PTEN occur in 30% of primary GBMs but only ~4% in secondary GBMs (Tohma et al., 1998). The PTEN protein can function as both a Serine/threonine /tyrosine protein-kinase and as a lipid phosphatase, with the phosphatase activity

seeming to be of primary importance in tumour suppression since many mutations have been found in this domain (Tamura et al., 1998; Maehama & Dixon, 1998). Phosphatidylinositol (3,4,5)-triphosphate (PIP3, a PI3K product) is a substrate of PTEN (Maehama & Dixon, 1998) and is activated in GBMs with PTEN deletions, which supports the view that PTEN could function as a TSG as well as a regulator of the PI3K-AKT pathway (Leevers et al., 1999; Newton, 2004). Enhanced AKT activity has been detected in PTEN-deficient tumours and cell lines from both humans and mice (Holland et al. 2000; Kondo et al., 2004). Overexpression of Akt as well as oncogenic Ras in mouse neural stem cells (NSC) leads to development of GBMs. Together, these observations suggest that the PI3K-AKT pathway is important in the aetiology of GBMs (Mischel & Cloughesy, 2003; Ghosh et al., 2005).

1-9-4 Candidate genes at other loci in 10q

Several candidate genes have been investigated in 10q in glioblastomas. STAM binding protein-like 1 (STAMBPL1), which is a cell surface receptor that transduces apoptotic death signals and is mapped in the same region as PTEN (10q23.3), and MXI1 MAX Interactor 1, which is a myc-C associated factor X (MAX) at 10q24-q25, appear to have been ruled out (Fults et al., 1998). Two candidates both mapped around 10q26-q27 are DMBT1 (deleted in multiple brain tumours) and MGMT. The MGMT gene is of particular interest. It encodes the DNA repair enzyme O-6-alkylguanine-DNA-alkyltransferase, which is responsible for protecting cells from alkylating agents. The enzyme efficiently removes methyl adducts at the O-6-position of guanine, which is an important target of alkylating agents (Pegg, 1990). It has been shown that the sensitivity to alkylating agents like BCNU correlates inversely with MGMT activity (Stupp et al., 2001; Gerson, 2002). The response of patients to BCNU is associated with an increase in overall survival rate (Esteller et al., 2000). Further more, aberrant hypermethylation of MGMT promoter is associated with loss of the MGMT protein, which is in contrast to retention of protein seen in the majority of tumours without hypermethylation (Esteller et al., 1999). Clinical trials have suggested that methylation of the MGMT promoter is predictive for better outcome in patients with malignant gliomas treated with alkylating agents such as temozolomide (Paz et al., 2004; Hegi et al., 2004), all

appearing to support the possibility of MGMT being a candidate TSG located at distal 10q.

1-10 The NSCs as probable cell of origin of primary GBMs

As shown by Holland et al., (2000), NSCs develop primary GBMs when subjected to overexpression of Akt and Ras oncogene. Other studies have subsequently shown that in vitro EGF but not PDGF can stimulate proliferation of neural stem cells (Miao et al., 2001; Mondal et al., 2004) while specific inactivation of Pten in mouse stem cells causes increased proliferation (Xiao et al., 2002; Fraser et al., 2004; Simin et al., 2006), which is thought to be as a result of shortening of the cell cycle of neural stem cells (Groszer et al., 2001). This effect of EGF signalling and PTEN in neural stem cell proliferation raises the possibility that primary GBMs might arise from neural stem cells (Ehtesham et al., 2004). Adult stem cells have been identified in humans (Temple et al., 1999; Rakic, 2002) and have been shown to express GFAP, which is a marker of astrocytes (Doetsch et al., 1999; Seri et al., 2001). This finding suggests that a close link might exist between the astrocytic lineage and adult neural stem cells (Quinn et al., 1999; Steindler & Laywell, 2002; Seidenfaden et al., 2006). Radial glia, which are traditionally considered to be astrocyte precursors, show characteristics of neural stem cells both in vitro and in vivo (Noctor et al., 2001). In mice it has been shown that neural stem cells are more susceptible to transformation than differentiated astrocytes (Holland et al., 1998; Holland et al., 2000). These observations are consistent with the view that the rapid growth of primary GBMs might be a consequence of transformation of neural stem cells (Miao et al., 2001). However primary GBMs arise mainly in patients who are older than 55 years of age (Kleihues et al., 2000; Maher et al., 2001), and neural stem cell-activity reduces with age (Gage et al., 2000). Both factors would appear to contradict the findings in mice. However, these apparently contradictory observations could be reconciled by several studies, one of which reported that oligodendrocyte precursors acquired characteristics of neural stem cells in response to certain exogenous stimuli (Kondo et al., 2000, 2004). Another study in which Ink4a/Arf $^{-/-}$ astrocytes as well as Ink4a/Arf $^{-/-}$ NSCs were transplanted into neonatal brains demonstrated that homozygous deletion of both Ink4a and Arf, but not of Ink4a, Arf, or p53 alone, results in "de-differentiation" of neonatal mouse astrocytes into neural stem

cells in response to Egf signalling (Bachoo et al., 2002). Overall, these observations support a model in which primary GBMs can arise from NSCs, which either exist in the adult brain, or can be generated from more differentiated, though perhaps not terminally differentiated cell types in response to oncogenic mutations (Sell, 1993, 2004; Scott et al., 2005).

1-11 Clinical features of astrocytomas

Several clinical and histological criteria are in use for classifying and grading malignant brain tumours, however, the one preferred by the majority of pathologists, and recommended by the World Health Organisation is known as the WHO grading system (Kleihues & Cavenee 2000). The WHO classification grades astrocytomas into 4 categories, Grades I – IV, in the order of increasing malignancy. WHO grade 1s, are pilocytic astrocytomas, and are considered to be largely non-infiltrating and as such circumscribed. Tumours of this grade can therefore be cured by surgical resection (Szymas et al., 2000). Grades II-IV are collectively known as diffuse astrocytomas. Tumours of malignancy grade II are known as fibrillary astrocytoma, grade III as anaplastic astrocytoma (AA), and grade IV as glioblastoma multiforme (GBM). WHO Grade 1 and II astrocytomas are considered as low-grade astrocytomas (LGAs) while Grades III and IV are high-grade astrocytomas (HGAs).

Fibrillary astrocytomas have a high tendency for malignant progression to higher grade, with the GBM as the most malignant phenotype-endpoint. Glioblastomas arising as a result of stepwise progression are termed as secondary GBMs as opposed to de novo GBMs that arise rapidly with no clinical history suggestive of a prior low-grade astrocytic tumour. These two broad subgroups of GBMs are associated with clinical manifestations and genetic profiles that appear to support the separation. The majority of secondary GBMs harbour alterations of TP53 and PDGFA, while most primary GBMs tend to have alteration of EGF and PTEN (Ohgaki, 2005). Evidence from both histological and molecular studies suggests the presence of intermediate categories of GBM, such as the giant cell and gliosarcoma types. While several histological

manifestations of glial tumour aberration, such as gliosarcoma, have been described, these are not specifically covered in this thesis.

1-11-1 Aetiology, Gender and Age distribution of HGAs

1-11-1-1 WHO Grade III astrocytomas

Little is known about their aetiology, but astrocytomas arising from inherited/familial syndromes appear to progress mostly from lower to higher grades (Arruda et al., 1995). WHO grade III astrocytomas typically affect young adults (Hirose et al., 2003), with a peak around age 45 years, but are very occasionally encountered in older patients. The ratio of males and females appears to be similar (Collins, 1995).

1-11-1-2 WHO Grade IV astrocytomas

GBMs predominantly affect adults with the peak incidence between 45 and 70 years (Ohgaki & Kleihues, 2005). However, interestingly, numerous cases of congenital GBM have been reported in the literature (for example, Lee et al., 1999; Castellano-Sanchez et al., 2005; Brat et al., 2007). Patients with secondary GBM are on average much younger than those of primary GBM (Ohgaki, 2005). Over 90% of GBMs are thought to be of the primary type (Ohgaki & Kleihues, 2007). Males appear to be affected more commonly than females, with male to female ration of 1.2:1 (Collins, 2004).

Presenting clinical features of a GBM vary with location in the cranial cavity and the stage of development. The usual presenting complaints are a history of epileptic seizure, often with non-specific neurological symptoms and no focal neurological abnormalities, headache and personality changes. Raised intracranial pressure may signify a larger space occupying lesion, a location in the vicinity of the brain stem or the posterior cranial fossa. It could signify imminent danger to life and constitutes a surgical emergency. In over 50% of the cases, patients present with a short duration of symptoms, typically lasting less than 3 months. Clinical suspicion of the diagnosis of glioma is confirmed by neuroimaging, with both CT and MRI providing useful clues. On CT scans, GBMs present as irregularly shaped lesions with an area of central

necrosis, which is surrounded by a contrast enhancing area corresponding to the highly vascularised zone. This rim is invariably infiltrated by tumour tissue (Thomas, 1998; Mitchell et al., 2005) and does not represent the outer limits of the tumour. A zone of vasogenic oedema may be present for a distance of two or more centimeters beyond this rim.

1-11-2 Locations of cerebral astrocytomas

Most supratentorial astrocytomas are located in the subcortical white matter, commonly in the frontal and temporal lobes. Tumours may infiltrate the adjacent cortex and basal ganglia. It is estimated that ~15% of GBMs spread via the corpus callosum to infiltrate the contralateral hemisphere. Imaging of such lesions usually shows the characteristic appearance of bilateral glioma that has been likened to the shape of a butterfly (Shimauchi & Kinoshita, 1989; Zakrzewska et al., 2007). GBMs do not commonly infiltrate the dura, however it does occasionally happen, in which case the result is widespread intracranial dissemination, which is known as gliomatosis cerebri (Marwin et al., 2005).

1-11-3 Macroscopic and microscopic features

The definitive diagnosis is made on examination of the tumour biopsy specimen, in which tumours appear pale yellow in contrast to the gray whitish normal appearance of the brain. Some areas may demonstrate signs of more recent (pink/bright red) or older (browning) haemorrhage. The histological diagnosis is based on recognition of the presence, or absence, of 4 morphologic criteria, namely: cellular and nuclear atypia (pleiomorphism), mitoses, endothelial proliferation and necrosis. Light microscopy of tumour sections stained in haematoxyline and eosin (H & E) usually show more intensely stained tumour tissue comprising a higher density of astrocytes of varying shapes and sizes (Revesz, personal communication).

In addition to the pleiomorphic appearance in the histology, AAs are distinguished from the LGAs by the presence of cells undergoing mitosis, and usually the astrocytes are far

less differentiated. On the other hand, a diagnosis of GBM is made if haemorrhagic and necrotic areas or evidence of formation of new vessels is found. Sometimes the histology is of a highly anaplastic cellular architecture, in which case, by accepted convention a tissue testing positive for GFAP is considered as of astrocyte origins (Johnson, 2002).

1-12 Clinical management

Conventional treatment of patients with high malignancy grade astrocytoma consists of surgery and radiotherapy (RT), while the standard treatment for recurrent tumours is chemotherapy (CT) with either lomustine alone, or in combination with procarbazine and vincristine (NHS Northern and Yorkshire, 2000). Although there are reports of benefits derived from adjuvant chemotherapy for patients with high malignancy grade gliomas, its role remains uncertain (MRC Brain Tumour Working Party, 2001).

1-12-1 Role of surgery

The invasive and widely infiltrative nature of high-grade astrocytomas makes curative resection impossible (Kelly et al., 1987. Thus, the impact of surgery is compromised because of the lack of a defined tumour edge and the close proximity of vital anatomical structures. Still, surgery has a vital role as the means for obtaining the biopsy specimen needed to establish the diagnosis, administering intratumoural wafers, as an aid to investigative procedures such as functional magnetic resonance imaging (fMRI) (Mueller et al., 1996; Nimsky et al., 2006), and may be employed in managing raised intracranial pressure arising from CSF circulatory block when surgical bypass procedures may offer temporary improvement in the quality of life for the patient (Thomas, 1998). Likewise, patients with large tumours in surgically accessible locations may derive temporary benefits from surgical tumour reduction (Mueller et al., 1996; Thomas, 1998; Nimsky et al., 2006).

1-12-2 Radiotherapy

Radiotherapy currently serves as adjunct to surgery in the treatment of high-grade astrocytomas. Normal CNS tissue can tolerate up to 60 Gy of radiation, which is below the threshold required to kill malignant glioma cells. Consequently, the risk of residual tumours remains high (Burnet et al., 2006). Stereotactic radiosurgery or radiotherapy, interstitial radiotherapy, boron neutron-capture therapy (Andrews et al., 2006), and several techniques that attempt to enhance the effect of radiotherapy, such as hypoxia-targeting pharmacoradiotherapy (Shibamoto et al., 2004), have so far provided only marginal improvements in survival.

1-12-3 Chemotherapy

The goal of chemotherapy is mainly to control tumour growth and to maintain, for as long as possible, a satisfactory quality of life of the patients (Castro et al., 2003). However malignant gliomas, in particular those of astrocytic origins, tend to be either initially or gradually chemoresistant (Shapiro et al., 1991). In part, the failure of chemotherapy may be due to the presence of the blood–brain barrier (Bigner, 1981), which may act as a physical barrier or operate different efflux pumps (Tamai et al., 2005; Girardin F, 2006) and so hinder the transport of chemotherapeutic agents into the CNS. Several chemotherapeutic agents are used alone, or in combination, as standard first-line drug treatments for primary malignant astrocytic tumours. The combinations of procarbazine, lomustine, and vincristine (Levin et al., 1995), or procarabazine, cisplatin, and vincristine (PCV) (Souhami et al., 2004), or carmustine (a DNA alkylating agent) alone, have demonstrated a significant prolongation of survival. All three have been found in several large randomised clinical trials to confer comparable levels of efficacy (Levin et al., 1995; Prados et al., 1999; Parney & Chang, 2003). However, recently, it has been shown that temozolomide (TMZ), when used as adjuvant chemotherapy in the prophylactic treatment of non-responsive, and inoperable primary as well as recurrent tumours, resulted in significant improvements in the symptoms and quality of life (Teixeira et al., 2003; Stupp et al., 2005), making this approach the new standard of care for GBM (Stupp et al., 2005).

1-12-4 Management of recurrent tumours

Recurrences of tumour in the same location are common and may be managed by re-operation where possible, additional radiotherapy and/or chemotherapy. Retinoic acid (Defer et al., 1997; Phuphanich et al., 1997; Wismeth et al., 2004; Parney and Chang, 2003) and interleukins (Kahlon et al., 2004) have demonstrated some benefits; the former promotes cellular differentiation in proliferating tissue (Parney et al., 2005) while the latter enhances immune response to tumour antigens (Kahlon et al., 2004).

1-12-5 Predictors of response to radiotherapy/chemotherapy

The two factors, namely the methylation status of the MGMT promoter, and the status of 1p and 19q in the tumour, in particular, combined deletion (codeletion), have recently emerged at important predictors of chemotherapy in some gliomas.

1-12-5-1 The role of MGMT in resistance to alkylating chemotherapy

Epigenetic silencing of the 06-methylguanine-DNA methyltransferase (MGMT) gene by promoter methylation has been recognized as an important factor for predicting outcome in GBM patients treated with alkylating agents such as TMZ and carmustine (BCNU) (Esteller et al., 1998; Hegi et al., 2004). The MGMT gene is located on CHR 10q26 and codes for an excision repair enzyme that removes alkyl-groups from 0-6-position of guanine, one of the targets of alkylating agents. The alkyl-group is transferred to the active site of the MGMT protein that thereby becomes irreversibly inactivated and subsequently degraded, requiring resynthesis. Although 06-methylguanine accounts for <10% of the lesions triggered by alkylating agents, it plays a major role as a trigger for cytotoxicity and apoptosis (Ochs & Kaina, 2000). If left unrepaired, e.g., due to epigenetic silencing of the MGMT gene or depletion of the MGMT protein by saturation of the process, 06-methylguanine forms mispairs with thymidine after replication that are recognized by the mismatch repair (MMR) system. However, since MMR is directed to the newly synthesised strand while the 06-MG resides in the template strand, the lesion is not repaired, but engages MMR in what is called futile repair cycle that eventually leads to cell death (Karran, 2001). Thus, suggesting that in addition to an inactivated MGMT gene, MMR-proficiency is an

important factor for response. Indeed, it has been reported cells deficient for MMR are 100 times more resistant to alkylating drugs even in the absence of MGMT (Stojic et al., 2004).

1-12-5-2 MGMT Promoter Methylation & Chemosensitivity of Gliomas

Methylation of the MGMT was found to have strong prognostic effect in a homogeneous cohort of patients treated with combination of radiotherapy and TMZ (Hegi et al., 2005). The impact of MGMT methylation is dependent on therapeutic modalities, for example, benefits were more apparent for patients treated with RT plus chemotherapy (CT), particularly when CT is administered during the cause of the RT (Criniere et al., 2007). In the absence of any treatment, MGMT methylation has no impact or may have a negative impact on survival (Komine et al., 2003; Criniere et al., 2007) while the impact on RT alone is less than that in patients receiving RT and TMZ (Stupp et al., 2005). The synergistic effect of RT + TMZ for patients with MGMT methylation is not clearly understood. CT is presumed to consume MGMT (Tolcher et al., 2003), whereas several in vitro studies suggested that RT upregulates MGMT (Grombcher et al., 1998) though this has not been confirmed by others (Hermisson et al., 2006). Older patients tend to have a higher rate of MGMT methylation (Rolhion et al., 1999). Since age is a very strong negative prognostic factor in GBM (Houilliet et al., 2006; Curran et al., 1993), it could mask the apparent positive effect of MGMT methylation in patients treated with alkylating agents, and it appears that the impact of MGMT methylation probably depends on type of alkylating drugs used for example, TMX vs. nitrosourea vs. other alkylating agents. The degree of methylation may also have a differential impact on chemosensitivity, while this is also dependent on the mismatch repair (MMR) system.

1-12-5-3 Deletion of 1p & 19q in the tumour

Chromosome 1p/19q codeletion as a predictor of chemosensitivity has mainly been studied in oligodendroglioma tumours and low-grade gliomas (LGGs) (e.g., Levin et al., 2006; Kaloshi et al., 2007; Kouwenhoven et al., 2006; Brandes et al., 2006; Walker et al., 2006). The survival of patients of anaplastic astrocytomas is dependent on the status

of 1p/19q in the tumour. Randomized studies carried out by the Radiation Therapy Oncology Group (RTOG) and the European Organization for Research and Treatment of Cancer (EORTC) were the first to reveal that tumours with the codeletion of 1p and 19q are more sensitive to chemotherapy, with 90-100% of the patients responding (Caincross et al., 1998; Van den Bent et al., 2003). Survival was also better in patients with tumours showing combined 1p/19q loss (Bauman et al., 2000; Smith et al., 2000). In Oligodendrogliomas, it was found that this 1p/19q codeletion is an early event in the tumourigenesis, which is mediated by an unbalanced translocation of 19p to 1q: der(1;19)(p10;q10) (Griffin et al., 2006; Jenkins et al., 2006). Patients with AAs (and OAs) having normal 1p/19q status survived ~ 2–3years, while patients whose tumours showed codeletion of 1p/19q survived 6–7years. Interestingly, the MGMT promoter methylation seems more frequent in tumours with combined 1p/19q combined loss, with 80% of tumours showing methylation (Mollemann et al., 2004).

1-13 Molecular Targeted Therapies

From a molecular perspective, malignant gliomas are extremely heterogeneous. Despite this variability, common alterations in specific cellular signal transduction pathways occur in most malignant gliomas. These include alterations in the p53 protein, the retinoblastoma susceptible protein (pRb), the Ras–Raf pathway and the phosphoinisitide-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway (Rich et al., 2004). Mutations within the p53 and pRb pathway lead to an inhibition of apoptosis and cell cycle arrest, and thereby remove the restrictions to proliferation (Vogelstein et al., 2000; Sherr, 1996). Alterations in the growth factor pathways, mediated by Ras and PI3K, are mostly responsible for proliferation of malignant gliomas (Wang et al., 1997). For example, an important signaling pathway in the development of malignant gliomas is mediated via the epidermal growth factor receptor (EGFR)/PI3K/Akt/mTOR pathway. This pathway is normally inhibited by PTEN, which is mutated in 30-40% of GBM (Wang et al., 1997; Stambolic et al., 1998). Another important signaling cascade, having a key role in angiogenesis, is the vascular endothelial growth factor (VEGF)-A pathway. The main receptors involved in VEGF-A signaling are VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (KDR, Flk-1). VEGFR-2 is a potent tyrosine kinase, which mediates endothelial cell signaling through activation of Ras, PI3K-and-protein kinase C-b pathways (Petrova et al., 1999). These

signalling pathways are responsible for the high vascularity of glial tumors, often with overexpression of VEGF-A (Plate et al., 1992; Chaudhry et al., 2001). For a number of these pathways, there are now available specific inhibiting agents (Rich et al., 2004), and clinical trials have focused mainly on the EGFR/PI3K, Ras and VEGF signaling cascades.

1-13-1 Epidermal growth factor receptor

EGFR is amplified in 40–50% and overexpressed in up to 60% of GBM (Ekstrand et al., 1992; Watanabe et al., 1996; Ohgaki et al., 2004). EGFR mutations occur in about 50% of EGFR-amplified GBM, most frequently affecting the extracellular domain with a deletion of exons 2–7 (EGFRvIII mutation) (Huang et al., 1997). The resulting variant receptor, EGFRvIII, is constitutively activated with continuously downstream signaling (Frederick et al., 2000; Aldape et al., 2004). This made the EGFR a logical target for clinical trials. The majority of these trials focus on targeting EGFR by small molecule tyrosine kinase inhibitors (TKIs) that act as competitive antagonists of the intracellular EGFR domain. ZD1839 (gefinitib/Iressa) and OSI774 (erlotinib/Tarceva), which both have specificity for EGFR/ HER1, are two EGFR TKIs currently under investigation (Prados et al., 2006; Reardon et al., 2006). Unfortunately, phase II data show that the overall efficacy of these agents in unselected patients with newly diagnosed or recurrent GBM is minimal (Rich et al., 2004; Raizer et al., 2004; Quan et al., 2005). The first correlative studies linked to these trials demonstrated that EGFR-expression, amplification or mutation does not predict response to EGFR TKIs (Lassman et al., 2005). A large study using two independent datasets suggested that coexpression of EGFRvIII and PTEN was statistically significantly associated with response to EGFR TKIs, with 60–80% of patients with this combination responding (Mellinghoff et al., 2005). However, a recent randomized European Organization for Research and Treatment of Cancer phase II study failed to establish clinically significant activity of erlotinib in unselected patients, and could also not confirm the responsiveness of GBM with combined EGFRvIII mutation and PTEN expression (Van den Brent et al., 2007).

1.13.2 Mammalian target of rapamycin (mTOR)

mTOR is a key downstream kinase in the PI3K/Akt/ mTOR pathway (Vivanco et al., 2002). PTEN gene mutations/deletions, occurring in 30–40% of patients with GBM, result in activation of the PI3K pathway (Wang et al., 1997). In preclinical models an enhanced sensitivity to mTOR inhibition was found in PTEN-deficient tumors (Neshat et al., 2001). Phase II results of two studies on the mTOR inhibitor CCI-79 (temsirolimus) in unselected recurrent glioma patients showed no significant efficacy (Galanis et al., 2005; Chang et al., 2005). In-vitro evidence of synergism between mTOR inhibitors and EGFR TKIs has resulted in concentrating efforts in clinical trials on combining these agents.

1-13-3 Platelet-derived growth factor receptor

Overexpression and activation of platelet-derived growth factor receptors (PDGFRs) play a role in cancer through autocrine stimulation, angiogenesis and control of tumor interstitial pressure (Ostman et al., 2004). PDGFR pathways promote glioma transformation and many gliomas show upregulation of PDGF (Lokker et al., 2002; Clarke ID, Dirks PB, 2003). Despite the rarity of PDGFR mutations in gliomas, these observations have prompted trials on PDGFR inhibition in malignant gliomas. The most frequently studied drug is imatinib (STI571), a small-molecule that is an inhibitor of PDGFR-a and b, c-kit, and the Bcl–Abl fusion protein. Phase II results of studies on imatinib monotherapy have so far showed no significant activity in recurrent gliomas (Raymond et al., 2003; Wen et al., 2006). However, interesting results were observed in initial studies on the combination of imatinib and hydroxyurea, resulting in 10–20% of patients responding, and 24–30% 6-month PFS (Dresemann G, 2005; Reardon et al., 2005; Desjardins et al., 2007).

1-13-4 Vascular endothelial growth factor receptor

Current strategies for targeting VEGF in malignant gliomas are directed to design and application of VEGFR-TKIs, VEGF antibodies and protein kinase-C-beta (PKC-b) inhibitors. Treatment of recurrent GBM with vatalanib (PTK787/ZK222584), a VEGFR-1 and VEGFR-2-TKI, showed disappointing results in two Phase I/II studies (Conrad et al., 2004; Reardon et al., 2004). In contrast, a recent study on the

combination of bevacizumab (Avastin), a VEGF-A monoclonal antibody, and irinotecan demonstrated activity in recurrent high-grade gliomas. In a group of 23 patients, a 63% response rate was found, with a median PFS of 23 weeks, whereas the 6-month PFS in GBM without this treatment was 38% (Vredenburgh et al., 2007). Finally, enzastaurin (LY317615), an inhibitor of activated protein kinase C-b2, is currently being tested in phase II studies in recurrent malignant gliomas. Preliminary results of a phase II study showed a 22% response rate but the 6-month PFS is still not reported. A randomized phase III study comparing enzastaurin to single-agent lomustine was prematurely closed due to expected failure to meet its endpoint, but combination studies of enzastaurin with TMZ and radiotherapy are ongoing.

1-13-5 Farnesyltransferase inhibitors

Farnesyl transferases are involved in signal transduction in the Ras pathway (Newton et al., 2003). The inhibitor, tipifarnib, had only limited activity as a single agent in recurrent gliomas, however, trials combining it with radiotherapy, TMZ and other targeted agents are underway (Cloughesy et al., 2005; 2006).

1-14 Prognostic factors

It is generally accepted that important prognostic factors in brain tumours are, 1) the age of the patient, 2) degree of neurological impairment (Karnofsky performance score, KPS), 3) extent of surgical resection of the tumour, 4) tumour type, 5) grade and 6) intracranial location (Rich et al., 2005). However regarding high-grade astrocytomas prognostic factors are as yet not clearly defined. The survival of patients with anaplastic astrocytomas, whose median survival is 3-5 years (Surawicz et al., 1998), is considerably better when compared with that of glioblastoma patients that on average is below 12 months. The mean survival times for GBM in the absence of treatment is around 17 weeks, 30 weeks following biopsy and radiotherapy, 37 weeks following partial surgical removal and radiotherapy, and 51 weeks following more extensive volumetric reduction procedure and radiotherapy (MRC Brain Tumour Working Party, 2001)

Some GBM patients are known to survive for longer periods, and are categorized as long-term survivors. In the context of GBM, survival of 3 or more years is considered to

constitute long-term survival (Burton et al., 2002). There is considerable uncertainty and disagreement among researchers on the proportion of GBM patients attaining long-term survival. More optimistic estimates have put the figure around 15 - 20% (Sant et al., 2003); others estimate it to be 2 – 5% (McLendon & Halperin, 2003; Senger, Caincross & Forsyth, 2003). Long-term survivors appear to have certain characteristics in common among which are, 1) the younger age and 2) favourable tumour indicators, such as less dedifferentiated tumours and tumours showing limited areas of necrosis, which may both be related to, 3) a favourable initial KPS (MRC Brain Tumour working Party, 2001).

There is significant genetic heterogeneity among tumour specimens from different patients with glioblastoma (von Deimling et al., 1993; Schmidt et al., 2002; Nutt et al., 2003), as there are intratumoural genetic differences (Burger Kleihues, 1989; Jung et al., 1999; Liang et al., 2005). There are suggestions that intratumoural genetic heterogeneities may contribute to differences in response to therapy (Hamstra et al., 2004). Mutations of the TP53 gene, and amplification, and rearrangement of the EGFR gene are common genetic alterations in patients with glioblastoma (Louis and Cavenee, 2001). Studies of their relationship with prognosis have yielded inconsistent results, which have been attributed to several causes, for instance, small sample sizes, inclusion of different tumour histologies, and lack of uniform treatment (Kraus et al., 2000). Nevertheless, in general, the prevailing view appears to be that the relationship of genetic alterations and prognosis in patients with GBM is complex and may be a function of the age of the patient (Simmons et al., 2001).

TP53 mutations, allelic loss of 1p (Caincross et al., 1998) and CDKN2A/p16 homozygous deletion (Ushio et al., 2003), which, previously, had either not been reported as having a prognostic value in astrocytomas (LOH 1p, CDKN2A (9p21), or over which there has continued to be a controversy (LOH TP53 (17p13) (Batchelor et al., 2004), and EGFR amplification (Barker et al., 2001), have each, individually, recently been reported to influence prognosis in high grade astrocytomas. However, as yet a consensus appears to exist only regarding the influence of TP53 mutations and LOH at 10q. Survival of patients with secondary glioblastoma is significantly longer

than that of those with primary glioblastoma. Absence of TP53 mutations and presence of LOH 10q are predictive of shorter survival. EGFR amplification, p16 INK4a homozygous deletion, and PTEN mutations are not associated with prognosis of glioblastoma patients (Ohgaki et al., 2004).

In some tumours, biological markers have been found that appear to be associated with age. For example, Ki-67 (MIB) labelling index and oestrogen receptor status, as well as the expression levels of EGFR, ERBB2, BRCA1 and BRCA2 in cancers of the breast (Garvin et al., 1997; Bogdani et al., 2002; Ito et al., 2001; Iwase et al., 2001), and thyroid (van Tol et al., 2001). Some studies have attempted to correlate age with biological markers in high-grade astrocytomas. For example, Kunwar et al., (2001) investigated 80 tumours of patients with anaplastic astrocytoma using comparative genomic hybridization, with the aim of assessing the relationship between cytogenetic alterations and clinical parameters. They observed cytogenetic alterations such as gain of 7p, and 19, and loss of 4q, to occur more commonly in older patients while loss of 11p was seen frequently in younger patients. In their study, the gain of 7p in older patients with AAs was a poor prognostic indicator.

1-14-1 Prognostic factors for glioblastomas

1-14-1-1 Small neoplastic cells

On the basis of 71 cases of glioblastomas, Burger PC & Green SB, in 1987, showed that patients with glioblastomas composed of homogeneous small neoplastic cells had shorter survival. However, in 2002, Schmidt et al., (2002), in a study of 97 glioblastomas, did not find differences in the survival of patients with glioblastoma with or without areas of better differentiation. This was supported in 2006 by the study of Homma et al., in which both univariate and multivariate analyses showed that small cells as the major cell type in glioblastomas were not predictive of survival. Burger et al., (2001) reported an association between the small-cell-phenotype, in glioblastomas, with EGFR amplification. They found that ~67% of exclusively small cell neoplasms, 32% of glioblastomas with both small and non-small cell areas, and 9% of non-small cell glioblastomas, had EGFR amplification. In 2004, Perry et al. also reported that

small cell glioblastomas showed frequent EGFR amplification (72%), as well as LOH 10q (100%), whereas none of the tumours (with small cells) showed loss of 1p/19q. The larger study of Homma et al., in 2006 subsequently confirmed the association of small cell phenotype and higher rate of EGFR amplification. In addition, they found that tumours with small cell glioblastomas had significantly more homozygous deletion of CDKN2A, but infrequent PTEN mutations, suggesting that their genetic basis may differ from that of other glioblastomas (Homma et al., 2006).

1-14-1-2 Oligodendroglial component

Homma et al., in 2006, also found that 20% of 403 glioblastomas investigated, showed focal areas composed of oligodendroglial cells. This result is similar to that of He et al., who, in 2001, found 17% of 142 GBMs contained foci of oligodendroglial cells. In the context of the oligodendroglial phenotype, several studies have reported longer survival of GBM patients with areas containing an oligodendroglial component (Nelson et al., 1996; Hilton et al., 1996). Hilton et al., in a study with 107 patients, showed that glioblastomas with an oligodendroglial component were associated with longer survival (median survival of 70 weeks) than those without (27 weeks). Similarly, Kraus et al., in 2001, reported the median survival of 12 patients with glioblastoma with an oligodendroglial component as 104 weeks, suggesting that these tumours are associated with better prognosis. This was contradicted by He et al., also in 2001, who reported that the age (median, 54 years) and survival (median, 11.5 months) of 25 patients with glioblastoma containing an oligodendroglial component, did not differ from those of patients with ordinary glioblastoma. Some studies reported that patients with glioblastoma having oligodendroglial features were younger than those with ordinary glioblastoma (Baker et al., 1996), but other studies failed to confirm this difference (Nelson et al., 1996; Kraus et al., 2001). The more recent and larger study of Homma et al., found that glioblastomas containing an oligodendroglial component developed in significantly younger patients. Univariate analysis revealed a tendency for longer survival of patients with glioblastoma containing an oligodendroglial component, and also showed that oligodendroglial components were significantly more frequent in glioblastomas that developed in long-term survivors (>18 months) than in short-term survivors (<6 months). On the other hand, multivariate analyses with adjustment for age

and gender did not show the presence of an oligodendroglial component as predictive of longer survival (Homma et al., 2006).

A related finding is the association between deletions of 1p/19q and the oligodendroglial cell type. Genetic analyses in 13 GBMs with an oligodendroglial component found LOH 1p in 3 (23%) with only one (8%) of these showing codeletion of 19q (Kraus et al., 2001) although He et al., in a study of 25 GBMs, reported a deletion of 1p and 19q in 40% and 60% of tumours with oligodendroglial cell type. The larger study (comprising 97 glioblastomas) by Schmidt et al., in 2002 found that some glioblastomas without oligodendroglial components also had LOH 1p/19q. However, the larger study of Homma et al., in 2006, found no correlation between oligodendroglial components and the presence of LOH 1p/19q in glioblastomas in both univariate and multivariate analyses.

1-14-1-3 Multinucleated giant cells

Multinucleated giant cells are frequently encountered in glioblastomas, with glioblastomas characterized by predominance of large, bizarre, multinucleated giant cells, being termed as giant cell glioblastomas (Peraud et al., 1999: Reis et al., 2001). These are rare variant, and are genetically characterized by frequent TP53 mutations (78%) and infrequent EGFR amplification (6%), (Peraud et al., 1999). The study of Homma et al., confirmed, in multivariate analyses, that the presence of >5% multinucleated giant cells was associated with frequent TP53 mutations and infrequent EGFR amplification.

1-14-1-4 The presence of necrosis

Patients with glioblastomas showing necrosis are significantly at higher risk of death than those without necrosis (Burger et al., 1987; Hammoud et al., 1996; Pierallini et al., 1998). The study, in 1996, by Hammoud et al., showed that the strongest prognostic variable was the amount of tumour necrosis on a preoperative scan. They found median survivals of 42, 24, 15, and 12 months, respectively for tumour-necrosis grades 0 (7

patients), I (11 patients), II (9 patients), and III (21 patients). Barker et al., in 1996, reported that 80% of patients in their study (275) who had supratentorial glioblastoma containing endothelial proliferation had tumour necrosis. Pierallini et al., found that patients with necrosis affecting >35% of the tumour had a significantly shorter survival time (Pierallini et al., 1998). The presence of necrosis was correlated with older age of patients and was associated with significantly shorter survival (Hammoud et al., 1996). The recent study of Homma et al., reported that the mean age of patients with glioblastoma with necrosis was 59.2 years, significantly older than those without necrosis (51.6 years), and survival of patients with glioblastoma with necrosis was 7.9 months, significantly shorter than those without necrosis (12.9 months). Multivariate analyses with adjustment for age and gender also indicated that the presence of necrosis is a significant predictive factor for poor survival of patients with glioblastoma.

1-14-1-5. Glioblastoma sub-type

These glioblastoma subtypes constitute distinct disease entities that affect patients at different ages, and evolve through different genetic pathways. Primary glioblastomas develop in older patients and typically show LOH on the entire chromosome 10, EGFR amplification /overexpression, and PTEN mutations (Burger et al., 2001; Ohgaki & Kleihues, 2005). Secondary glioblastomas develop in younger patients and typically contain TP53 mutations as an early alteration, and LOH 10q as a late event (Burger et al., 2001; Ohgaki & Kleihues, 2005). Histological features may also differ between primary and secondary glioblastomas. Large ischemic necrosis is significantly more frequent in primary glioblastomas than secondary glioblastomas (Tohma et al., 1998). The study of Homma et al., confirmed these results, and further showed a tendency for a more frequent oligodendroglial component in secondary than in primary glioblastomas, whereas small cell phenotypes were observed at similar frequency in both glioblastoma subtypes. In addition, they observed a significant association between the presence of necrosis and absence of TP53 mutations in glioblastomas. This can probably be explained at in part by the infrequent occurrence of TP53 mutations in primary glioblastomas that more frequently show necrosis.

1-14-2 Long-term survival of patients with glioblastoma

Survival is still extremely poor despite advances in surgical and clinical neurooncology. In a meta-analysis of 12 randomised clinical trials, the overall survival rate of patients with high-grade glioma was 40% at 1 year (Stewart, 2002). At the population level, survival of patients with glioblastoma was even worse, observed survival rates were 42.6% at 6 months and 17.7% at 1year (Burger et al., 2001). However, some patients survive longer, and several studies have focused on identification of histological and genetic features of glioblastomas from long-term glioblastoma survivors. Scott et al (1998) reported that 5 glioblastomas in patients who survived for >3 years after diagnosis did not show differences with respect to the presence of necrosis, vascular proliferation, lymphocytic infiltration, nuclear pleiomorphism, and nuclear size compared with 286 cases of all glioblastomas. Burton et al (2002) reported that the presence of necrosis or microvascular proliferation was not different between long-term (>3years) and short-term glioblastoma survivors (<1.5 years). McLendon et al (2003) reported that intermediate fibrillary components were more frequent and small cell components less frequent in 17 cases of glioblastoma from patients who survived for more than 5 years.

Burton et al., (2002) compared genetic alterations in glioblastomas from long-term survivors (>3 years; 41 patients) and those from short-term survivors (<1.5 years; 48 patients). Nuclear p53 expression was significantly more frequent in the long-term survivor group (85% vs 56%). Kraus et al (2000) showed no differences in TP53 mutation, PTEN mutation, CDKN2A deletion, EGFR overexpression among 21 long-term (>24 months), and 21 short-term (<6months) survivors of glioblastoma. Homma et al., in their study of 2006, found that patients with glioblastoma who survived more than 18 months were younger (mean, 50 years) and their tumours contained oligodendroglial components more frequently (27% vs 14%). However, LOH 10q was the only genetic alteration that tended to be less frequent in glioblastomas from long-term survivors (Homma et al., 2006).

1-15 Cytogenetic detection of aberrations in astrocytomas

Cytogenetic techniques, designed for studying gross structural alterations in cancer chromosomes have been applied with improvements over the past thirty-five years to a level where they are now very sensitive (Kearney, 2001; Subramonia-Iyer, 2007). The original cytogenetic technique was called karyotyping (Wahl et al., 1982), and required culturing cells. 'Chromosome spreads' prepared from cells that are induced to undergo mitotic arrested at metaphase were examined visually. This procedure was initially applied to the study of chromosomal abnormalities in haematological malignancies (Kallioniemi et al., 1995) and was instrumental in revealing many alterations that identified candidate genes implicated in these malignancies. While karyotyping was generally successful in haematological malignancies, in which cells can be grown with relative ease, it proved difficult to apply to solid tumours. Astrocytomas are far more difficult to grow, and are often unable to yield cells with adequate metaphase spreads for analysis by conventional banding techniques. An improvement from karyotyping led to fluorescence in situ hybridisation (FISH) techniques (Nacheva et al., 1998). FISH techniques require the use of specific genetic markers (chromosome probes) to map chromosome regions that are altered in tumour cells. FISH procedures are currently available in a number of refinements that may require the use of metaphase or interphase nuclei (Jacobsen et al., 2000). The use of interphase nuclei is intended to overcome the difficulties encountered in growing cells from some tumour. More recent refinements of the FISH techniques have enabled painting of whole chromosomes, allowing easy identification (Kubota et al., 2001), which has made it even easier to detect balanced translocations in chromosomes (Youings et al., 2004). However, in general, the growing of cells outside the body is associated with several potential drawbacks. In addition to difficulties encountered in growing cells from some solid tumours, a major drawback is the uncertainty about the true representation of original tumour in cell cultures. It is possible in some instances that the clone of cells that may grow might not be representative of the original tumour. In addition FISH procedures require specific genetic markers (probes) to map aberrant chromosome regions. Furthermore, both karyotyping and FISH procedures are unsuitable for studying the entire genomes of cancer cells. Two derivations of the conventional FISH procedure that are termed as Multi colour Fluorescence In situ Hybridisation (M-FISH) and comparative genomic hybridisation (CGH) have made it possible to study the entire

genome in a single hybridisation experiment (Mohapatra et al., 1997; Kearney L, 2006), which has singled them our as invaluable procedures for whole genome analyses.

Figure 1-7 (taken from Kearney L, 2006) shows the combination-labelling scheme for the M-FISH procedure using five spectrally separable fluorochromes. Each chromosome is labelled with a unique combination of fluorochromes: five chromosomes are labelled with a single fluorochrome, ten chromosomes with two fluorochromes, and nine chromosomes with three fluorochromes, respectively. The 'Colour' column shows the pseudocolour assigned to each unique colour combination.

1-15-1 Comparative genomic hybridisation (CGH)

CGH was developed by Kallioniemi et al., in 1992, and has since contributed a great deal to genome-wide investigation of solid tumours. The CGH method involves simultaneous hybridisation, onto a slide containing normal male lymphocyte metaphase chromosomes, separately labelled DNAs from two sources, one from a patient and the other, normal DNA, serving as reference. Unlike karyotyping, CGH requires only genomic DNA from a tumour and therefore has a wider application. For example, it can be applied to both fresh and paraffin-embedded tissue specimen (Kallioniemi et al.

1992, Speicher et al. 1993, Isola et al. 1994). Furthermore, even very small tumour samples can be studied by the CGH technique after amplification of the tumour material using any of the available whole genome amplification procedures such as multiple displacement amplification (MDA) (Dickson et al., 2005), or degenerate oligonucleotide primed polymerase chain reaction (DOP-PCR) (Speicher et al 1993, Hirose et al.2001). On the downside, the CGH method only detects chromosomal imbalance, missing out balanced chromosomal translocations, inversions, or small intragenic rearrangements (Kallioniemi et al, 1994). An additional limitation of the CGH procedure is the narrow range of sensitivity. It can only reliably detect large-sized structural alterations, those estimated to be in the order of 10 to 20 million bases (MB). Aberrations smaller than 10 MB easily escape detection. In particular, chromosomal aberrations that are present in low frequency, amplifications smaller than 2 megabases (Mb) and deletions smaller than 5 MB are easily missed by the CGH method (Forozan et al. 1997).

CGH has been widely used to investigate chromosomal aberrations associated with progression and clonal expansion in a variety of tumours including astrocytomas (Bigner et al. 1999), and has been successfully used in identifying novel genes involved in tumourigenesis (Hemmilinki et al., 1997). Some CGH studies focus on identifying recurring chromosomal aberrations and associations with clinical, pathological or prognostic factors (Vettenranta et al. 2001). A typical cytogenetic pattern observed in astrocytic tumours has been a direct correlation of the number of chromosomal abnormalities with increasing histopathological malignancy (Goussia et al., 2000).

1-15-2 METAPHASE CGH: BRIEF HISTORY OF THE PROCEDURE

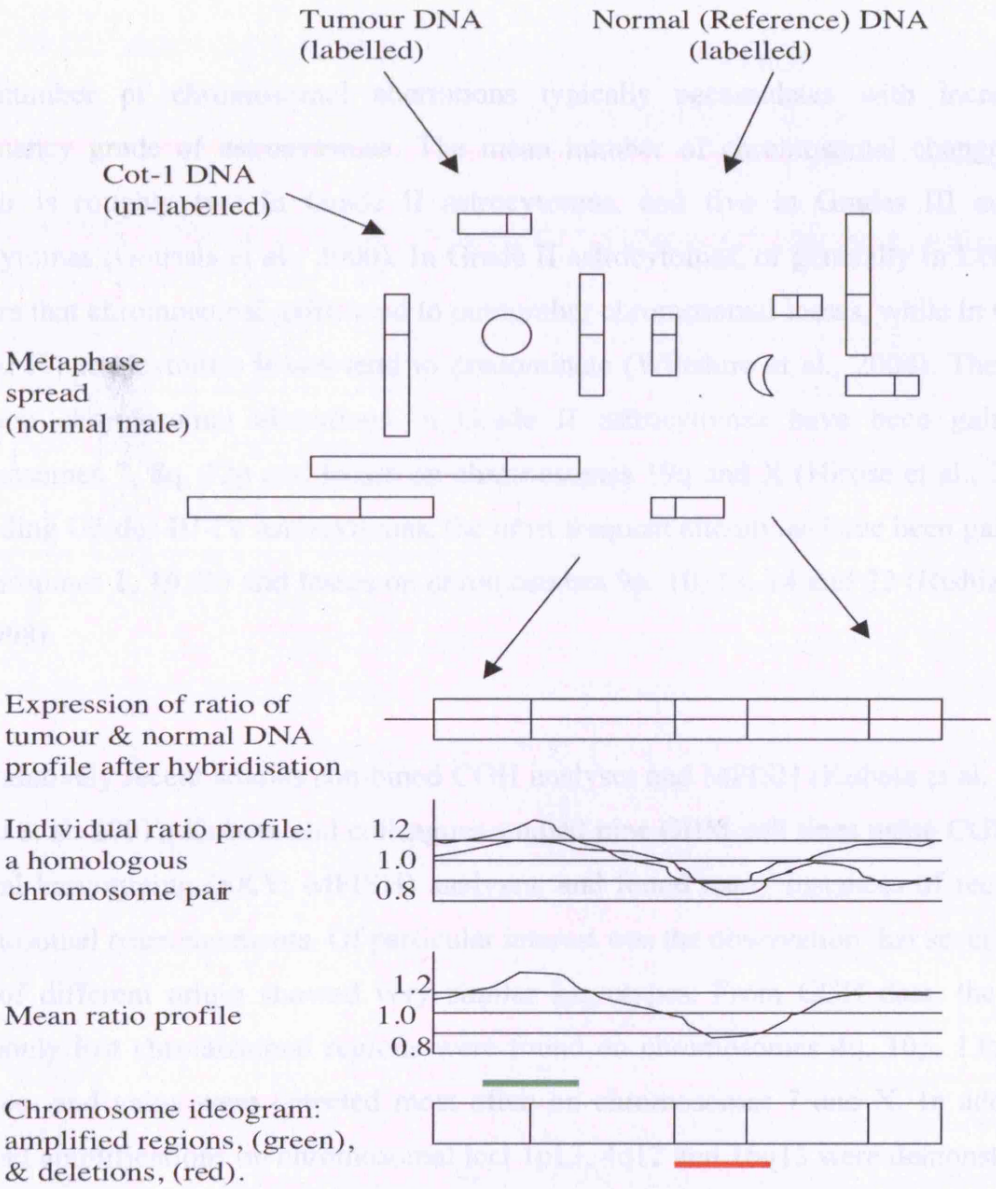
Comparative genomic hybridisation is used to examine an entire genome for changes in DNA sequence copy number - gains (e.g., amplification) and losses (e.g., deletions) - using extracted tumour DNA or other specimen DNA as a probe. Unlike conventional cytogenetic analysis, such as metaphase FISH, CGH does not require a previous knowledge of genetic aberrations or require a complex and extensive series of

chromosome-/ or loci-specific probes to fully evaluate the extent of genetic gains or losses in a genome. CGH has been used to examine chromosomal aberrations in several types of solid tumours including brain tumours. The results from these studies were in agreement with previously reported genetic aberrations or were verified using procedures such as FISH, karyotyping or PCR analysis. Additionally, CGH analysis has highlighted several unreported aberrations (Kallioniemi et al., 1995). Used to detect numerical chromosomal aberrations in prenatal samples, CGH identified the origins of extra and missing chromosomes in cases that could not have been resolved by karyotyping (Bryndorf et al., 1993). Classical cytogenetics is limited in the analysis of solid tumours because of the technical difficulties in obtaining metaphases and in interpreting karyotypes that are often complex. Furthermore, cells that proceed to mitosis may not be representative of the tumour. On the other hand comparative genomic hybridization uses the genomic DNA directly extracted from the tumour samples containing >50% tumour cells, ideally eliminating the need for cell culture. CGH has been successfully used to examine formalin-fixed tumour samples (Burger et al., 2001). This opens to analysis a variety of archival material that could not be examined by other cytogenetic methods. The technique of CGH is based on a two-colour competitive fluorescence in situ hybridisation of differentially labelled tumour and reference DNA to normal metaphase chromosomes (Kallioniemi et al., 1995), see illustrated below. The ratio of fluorescence intensities reflects the relative copy number in the tumour DNA compared to the normal DNA. Amplifications and deletions in the tumour will give distinct signals, therefore revealing the chromosomal positions of specific alterations (Schlegel, 1996).

In order to determine chromosomal location of copy number alterations (CNA) that may contribute to development of astrocytomas, CGH was performed on DNA extracted from various sources as already stated. The CGH procedure involved simultaneous hybridization of two genomic samples to normal metaphase chromosomes in the presence of Cot-1 DNA, which serves to block the repetitive sequences (Traut et al., 1999). Tumour DNA (alternatively referred to as test DNA) was usually labelled with Spectrum Green dUTP (e.g., fluorescein) and the normal (reference DNA) is then labelled with SpectrumRed dUTP (e.g., Texas Red). The basic assumption in CGH is that the hybridisation kinetics of the test and the reference DNA are independent, so the

ratio of binding of the DNA to 'target' (metaphase) chromosomes is proportional to the ratio of the copy numbers of the sequences in the DNA samples at a specific locus (Piper et al., 1995). The relative fluorescence intensities of the test DNA to the reference DNA, hybridized onto normal metaphase chromosomes, were used to determine the regions of changed copy number. At least 10 metaphases were analysed in each experiment as recommended by du Manoir et al., (1995)

Figure 1-8 Diagram of the CGH technique



1-15-2-1 Typical CGH findings in astrocytomas

Most grade II and III astrocytomas have been reported to have a few aberrations on CGH examination. Typically, biopsies show losses on the X chromosome (Schrock et al., 1996), while cell cultures of the majority of tumours may have additional alterations (Magnani et al. 1994). A few cases of grade III astrocytomas have been noted to have trisomy of chromosome 7 and losses on chromosomes 6, 10, 22, X and Y as well as structural abnormalities involving the short (p-) arm of chromosomes 1 and 9 (Goussia et al., 2000; Ueki et al., 2002).

The number of chromosomal aberrations typically accumulates with increasing malignancy grade of astrocytomas. The mean number of chromosomal changes per tumour is roughly two in Grade II astrocytomas, and five in Grades III and IV astrocytomas (Goussia et al., 2000). In Grade II astrocytomas, or generally in LGAs, it appears that chromosomal gains tend to outnumber chromosomal losses, while in Grade III and IV astrocytomas losses tend to predominate (Wiltshire et al., 2004). The most common chromosomal alterations in Grade II astrocytomas have been gains on chromosomes 7, 8q, 12p and losses on chromosomes 19q and X (Hirose et al., 2003). Regarding Grades III-IV astrocytomas, the most frequent alterations have been gains on chromosomes 1, 19, 20 and losses on chromosomes 9p, 10, 13, 14 and 22 (Nishizaki et al., 1998).

Two relatively recent studies combined CGH analyses and MFISH (Kubota et al. 2001, Squire et al. 2001). Kubota and colleagues studied nine GBM cell lines using CGH and spectral karyotyping (SKY; MFISH) analyses, and found many instances of recurrent chromosomal rearrangements. Of particular interest was the observation that several cell lines of different origin showed very similar karyotypes. From CGH data, the most commonly lost chromosomal regions were found on chromosomes 4q, 10p, 13q, 14q and 18q, and gains were detected most often on chromosomes 7 and X. In addition, frequent amplifications on chromosomal loci 1p13, 4q12 and 16q13 were demonstrated. Notably, data from SKY analysis demonstrated that regions of low-level DNA amplification tended to harbour translocations and/or insertions at a high rate (Kubota et al. 2001). The study of Squire and colleagues, on the other hand, which utilized 16 cell-

lines, of which ten were of glial tumours, showed that the chromosomes affected most often by translocation events were 1 and 10. Other chromosomes that were less implicated in translocations were chromosomes 3, 5, 7 and 11. The most common alteration detected by CGH was gain on chromosome 7.

1-16 DNA microarrays

Following the introduction of DNA microarrays approximately 10 years ago several versions have come into use and have provided a rapid method for exploring the tumour genome. Arrays are designed to act as gene-specific hybridisation targets which has made it possible, in the course of a single hybridisation experiment, for clones representing thousands of genes to be assessed for copy number aberrations in the same tumour sample (Ramsay, 1998; Fiegler et al., 2007). In addition, it is possible to compare two biological samples by simultaneously hybridising a mixture of two independently labelled probes to the same microarray (Schena et al. 1996). Most experiments have utilised arrays of DNA fragments or large genomic clones (Solinas-Toldo et al., 1997; Ekong et al., 2004; Fiegler et al., 2006). Microarray-based CGH has ~ 20-fold higher mapping resolution than conventional metaphase CGH (Pollack et al. 1999), depending on spacing of clones on the array.

From the point of view of clinical application, a potentially important adaptation of array technology is the incorporation of whole tissue into what has been termed as "tissue microarray", which is intended to achieve parallel detection of DNA, RNA and protein targets in multiple tumours (estimated in hundreds) in a single hybridisation procedure (Kallioniemi et al. 2001). Tissue arrays are constructed by bringing small circular tissue biopsies from different tumours into a single paraffin block. So far up to 1000 samples can be applied to one tissue-array-block (Hocquette, 2005). However, the high-throughput nature of microarray experiments imposes numerous limitations, which apply to simple issues such as sample acquisition and data mining, to more complex issues that relate to the methods of biostatistical analysis required to analyze the enormous quantities of data obtained. Furthermore, genomic arrays for detection of DNA copy number aberrations are also unable to detect aberrations resulting in

balanced rearrangements, and are as yet unable to identify aberrations in chromosomal regions that are enriched in highly repetitive DNA sequences, duplications, polymorphisms and copy number variations (CNV) (Carter et al., 2002; Ekong et al., 2004; Redon et al., 2006; Fiegler et al., 2007).

1-17-1 Use of microarrays to detect gene expression

The use of microarray techniques, especially cDNA microarray, for gene expression profiling of various cancers (for example, Mischel et al., 2004; Neben et al., 2004; Ge et al., 2005), have yielded valuable results, demonstrating the potential for their clinical usefulness. A study of expression profiles of tumours of the breast (van Veer et al., 2002) and gliomas (Mischel et al., 2003) using cDNA arrays found a correlation with the clinical outcome of patients, thus demonstrating the clinical utility of cDNA microarrays. A number of studies of microarray-based analyses of astrocytomas have been reported. In a 1999 study by Fuller et al., cDNA microarray analysis of 588 known genes revealed the expression of insulin-like growth factor receptor binding protein 2 (IGFBP2; 7p13-12) in GBMs, but not in anaplastic astrocytomas. Another study of the gene expression profile of 1176 known cancer-associated genes in 11 Grade II astrocytomas, by Huang and colleagues, in 2000, demonstrated significant expression changes in 24 genes. The expressions of tissue inhibitors of metalloproteinase 3 (TIMP3; 22q12.1-13.2), cellular MYC (C-MYC; 8q24), epidermal growth factor receptor (EGFR; 7p12), non-metastatic cell 3 (NME3; 16q13.3), 2-4-dienoyl CoA reductase 2 (NME4; 16p13.3) and serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2, or glial-derived neurite promoting factor, protease inhibitor 7 (SERPINE; 7q21.3-22) were detected in the majority of Grade II astrocytomas but not in normal brain tissue. In addition, the AADH, secreted protein, acidic, cysteine-rich (SPARC; 5q31-33), low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor) (LRP; 12q13-14), platelet derived growth factor receptor-a (PDGFRA; 4q12), 60S ribosomal protein L5, pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1) (PTN; 7q33), deltex 3-like or B-lymphoma- and BAL-associated protein (DTX3L; 3q21.1) were demonstrated to be upregulated more than 2-fold in 20-60% of Grade II astrocytomas. Meanwhile, interferon induced transmembrane protein 1 (IFITM1;

11p15.5), CDC-like kinase 1 (protein kinase CLK), teratocarcinoma-derived growth factor 1 (TDGF1; 3p21-31), bridging interactor 1 (BIN1; 2q14), G-protein receptor binding 2 associated (GRB2-associated) binding protein 1 (GAB1; 4q31.1), tyrosine-protein kinase receptor TYRO3 precursor (TYRO3; 15q15.1-21.1), lactate dehydrogenase A (LDHA; 11p15.1), adducing 3, guanylate kinase 1 (GUK1; 1q32-41), cell division cycle 10 (CDC10), and keratin 8 (KRT8; 12q13) were down-regulated more than 50% in the majority of the tumours (Huang et al. 2000).

Similarly, distinctive molecular profiles of low-grade and high-grade astrocytomas were demonstrated using oligonucleotide-based microarray analysis of approximately 6800 genes (Rickman et al. 2001). Out of the above panel, a total of 378 genes differed in their expression patterns between Grade II astrocytomas and normal brain samples, while 1305 genes had differences in expression levels between GBMs and normal brain tissue samples. A comparison of the expression profiles of GBMs with Grade II astrocytomas, found 183 genes expressed at a higher level and 149 genes at a lower level. Many of the genes found upregulated in GBMs encoded proteins that are involved in cell proliferation or cell migration (Rickman et al. 2001). A second study of oligonucleotide-based microarray on four GBMs identified several downregulated ion and solute transport-related genes (Markert et al. 2001), while several others: aquaporin-1, solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3; 12p13.1), tumour necrosis factor (ligand) superfamily, member 11 or osteoporin (TNFSFII; 13q14), nicotinamide N-methyltransferase (NNMT; 11q23.1), murine double minute 2 (MDM2; 12q13-14), granulin (GRN; 17q21.32), tumour protein p53 binding protein, 1 or cytokine and P53 binding protein (TP53BP1; 15q15-21), and macrophage migration inhibitory factor (MIF; 22q11.23) were found to be upregulated.

Several GBM short-term cell cultures (STCC) and solids tumour samples (biopsies) were analysed by array CGH to determine expression levels of 58 target oncogenes (Hui et al. 2001). Their study revealed high-level amplification of CDK4 (12q13), glioma-associated oncogene homolog 1 (GLI; 12q13.3-14.1), MYCN (2p24.3), MYC (8q24), MDM2 (12q13-14) and PDGFRA (4q12), and frequent gains of phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA; 3q26.3), EGFR (7p12), chromosome segregation 1-like (CSE1L; 20q13), NRAS (1p13.2), MYCN

(2p24.3), Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog (FGR; 1p36.2-36.1), estrogen receptor 1 (ESR; 6q24-27), ATP-binding cassette, sub-family B (MDR/TAP), member 1 (ABCB1; 7q21.12). This suggests involvement of these genes in GBM tumourigenesis.

1-18 Whole Genome Amplification (WGA)

Metaphase CGH requires large amounts of starting DNA (typically 0.2-1ug; Wells et al., 1999) for the labeling (nick-translation) reaction. However, several tumour samples for this study had small amounts of starting DNA and therefore required amplification by a procedure capable of achieving uniform whole genome replication. The concept of whole genome amplification (WGA) arose as the polymerase chain reaction (PCR) was adopted to replicate regions of genomes that are of biological interest (Telenius et al., 1992; Zhang et al., 1992) and has been successfully used to amplify minute amounts of DNA (for example, from a single cell (i.e., ~6pg) for CGH to assay chromosomal mosaicism in preimplantation embryos (Wells et al., 1999; Wells & Delhanty, 2000).

Several approaches are used to achieve WGA, among them *in vivo* amplification in Escherichia coli hosts, polymerase chain reaction (PCR), and isothermal rolling circle DNA replication. Ideally, a WGA procedure should avoid bias (i.e., preferential amplification of parts of the target genome) while achieving accurate replication of the original genome sequence. A number of studies have demonstrated that both DOP (degenerate oligonucleotide-primed) PCR (DOP-PCR) and MDA (multiple displacement amplification), an isothermal (non-PCR) strand-displacement amplification procedure) meet these criteria (for example, Wells et al., 1999; Wells & Delhanty 2000; Detter et al., 2002; Hawking et al., 2002; Hughes et al., 2005). Amplification products obtained using DOP-PCR are typically between 300bp-3Kb and therefore can be labelled without enzyme digestion prior to use in metaphase CGH. On the other hand, rolling cycle amplification such as MDA produce DNA of ~10Kb, which has to undergo enzyme digestion during labelling to produce fragments suitable for hybridization.

One immediate advantage of MDA results from the ø29 DNA polymerase error rate, which is reported to be ~1 in 10^6-10^7 (Esteban et al., 1993) in contrast to Taq DNA polymerase (used in DOP-PCR), which has an error rate of 3 in 10^4 (Eckert & Kunkel, 1991) or 1.6 in 10^6 for Pyrococcus furiosus (Pfu) DNA polymerase (Lundberg et al., 1991). Thus, although both DOP-PCR and MDA are generally considered suitable for generating products that are non-biased and an accurate replication of the original genome sequence, for this study, the need to generate DNA of a higher size as well as consideration of the slight advantage of lower error rate reported by earlier studies, led us to choose the MDA procedure over DOP-PCR for amplifying samples with inadequate DNA.

1-19 Aims of this thesis

High-grade astrocytomas, in particular glioblastoma multiforme (GBM), which is the most common primary tumour of the brain, continue to present challenges in their management. Primarily due to the infiltrating nature, there is no effective treatment for glioblastomas, while on the other hand, relatively little is known about the processes by which they develop. The way forward appears to be in the design of effective novel therapies and treatments for GBM, which requires research, to identify cellular and molecular mechanisms that cause GBMs. Modelling of astrocytomas by genetic manipulation of mice suggests that deregulation of pathways that control gliogenesis during normal brain development, such as the differentiation of neural stem cells (NSCs) into astrocytes, might contribute to GBM formation. These pathways include growth factor-induced signal transduction routes, and processes that control cell cycle progression such as the p16-CDK4-RB and the ARF-MDM2-p53 pathways. The expression of several components of these signaling cascades has been found altered in GBM, and recent data indicate that combinations of mutations in these pathways may contribute to GBM formation, although the exact mechanisms are still uncertain. High malignancy grade astrocytomas typically display a wide variety of molecular aberrations and biological behaviours, which have been used to classify them as primary (de novo) or secondary (progressive). The assumption seems to be that astrocytes from which the tumours originate are homogeneous. However individual tumours within the two established categories are known to display a wide range of molecular differences as well as intratumoural heterogeneity. Several possibilities could account for this phenomenon; for example, a straightforward possibility could be the random genomic rearrangements that arise as tumours evolve. An alternative possibility is the likelihood that certain aberrations could be associated with molecular heterogeneity in astrocyte subpopulations from which tumours originate. This view is supported by findings of various investigators, showing that there is regional restriction of astrocyte progenitors in the CNS. In order to shed light on this matter the objective of this project was to establish the frequency of copy number alterations, and their extent and chromosomal locations in a group of relatively young adults diagnosed with high malignancy grade astrocytomas. In the longer term, it is hoped this data can be used as a framework for investigating a relationship of various copy number alterations and prognosis.

CHAPTER 2 MATERIALS AND METHODS

2-1 THE RESEARCH PROPOSAL

This study involves 32 tumours of 31 patients (table 2-1) diagnosed and treated for high malignancy grade astrocytomas.

Table 2-1

1	Anaplastic astrocytomas (AA) – WHO Grade 3			
	1.1	Solid tumour samples (biopsies) (AS)		
		ID	**Gender**	**Age (Yrs)**
		1.1.1 AS24	F	48
		1.1.2 AS2641	F	40
		1.1.3 AS2706	F	38
		1.1.4 AS2721	M	46
		1.1.5 AS2745	M	31
	1.2	AA Short-term cell cultures (STCC) (AC)		
		1.2.1 AC545	Male	38
2	Glioblastoma multiforme (GBMs) – WHO Grade 4			
	2.1	Solid tissues (biopsies) (GBS)		
		2.1.1 GBS11	M	53
		2.1.2 GBS1575	F	61
		2.1.3 GBS1625	M	60
		2.1.4 GBS2051	M	44
		2.1.5 GBS2093	M	53
		2.1.6 GBS2126	M	41
		2.1.7 GBS2409	F	41
		2.1.8 GBS2532	M	50
		2.1.9 GBS2858	M	54
		2.1.10 GBS3044	M	56
	2.2	DNA of GBM biopsies amplified by Multiple Displacement Amplification (MDA)		
		2.2.1 GBS1595m	F	67
		2.2.2 GBS1926m	M	N/A
		2.2.3 GBS2650m	M	44
		2.2.4 GBS2687m	M	48
		2.2.5 GBS2848m	M	46
	2.3	DNA from GBM Short-term Cell Cultures (STCC) (GBC)		
		2.3.1 GBC160	M	40
		2.3.2 GBC1397	M	38
		2.3.3 GBC1510	M	50
		2.3.4 GBC1706	M	47
		≠*2.3.5 GBC1724	M	44
		2.3.6 GBC1752	M	44
		2.3.7 GBC2394	M	47
		2.3.8 GBC2685	M	48

2.4	DNA from GBC amplified by MDA			
	≠2.4.1	GBC1612m	M	44
	2.4.2	GBC1719m	F	69
	2.4.3	GBC1760m	F	57
	≠*2.4.4	GBC1724m	M	44

Explanation for table 2-1
List of tumour samples and patients investigated in this study. There was no constitutional blood or normal DNA to serve as a match for the tumour. F, Female, M, Male, N/A not available, *, same tumour sample, ≠, same patient

Continuation of text...

Laboratory data were acquired from 19 frozen biopsies and 14 short-term cell cultures obtained from the tumour archive held at the Institute of Neurology's Department of Neurosurgery. The Institute of Neurology is a department of University College London and is affiliated to the National Hospital for Neurology and Neurosurgery. A number of tumour samples were of patients previously enrolled in the MRC-BRO5 multi-centre Clinical Trial (MRC Brain Tumour Working Party, 1990; 2001), which was conducted between 1985 and 1997. Additional specimens were from patients managed in the period after the clinical trial, and were provided by several previously participating hospitals, such as the Preston NHS Trust, Addenbrooke, and several other hospitals. The research proposal was reviewed and approved by the Education Sub-committee of the Academic Board of the Institute of Neurology.

2-1-1 BACKGROUND

This study investigated chromosomal *copy number abnormalities* (CNAs) in 33 high malignancy grade astrocytomas, primarily using the method of *comparative genomic hybridisation* (CGH). Seven tumour specimens were investigated further by array CGH, however one particular glioblastoma was investigated more extensively, largely for purposes of optimizing the various experimental procedures and also to illustrate several other fluorescence *in situ* (FISH) procedures, such as multicolour FISH (MFISH), and microarray CGH. Each tumour specimen was assessed for chromosomal copy number alterations. Survival data were compared in patients with and without losses and gains in chromosomal material, stratifying for only age, sex and grade, which were the clinical variables for which data were available.

2-1-2 CONFIRMATION OF HISTOLOGICAL DIAGNOSIS

Although the tumour classification and grades at the time of recruitment into the BRO5 trial were determined by a Pathology Reference Panel comprising three pathologists (MRC Brain Tumour Working Party, 2001), in the course of this study each specimen (histology slide from previous biopsies) of patients recruited into the study was reviewed by Professor Tamas Revesz and myself. Our objective was to identify the blocks with sufficient amount of tumour, and to reconfirmation the diagnosis. Professor Revesz of the Department of Neuropathology at the Institute of Neurology is a consultant neuropathologist and was previously one of three members constituting the Pathology Reference Panel for the BRO5 Clinical Trial.

2-1-3 PATIENTS AND SAMPLES

The study involved 31 patients with high-grade astrocytomas. Two tumour samples (GBM/C1612, GBM/C1724) were from one patient. Tumour DNA was available as frozen solids in 19 cases and as short-term cell cultures in 14. The solids comprised 5 AAs and 15 GBMs. Nine starting DNA samples were inadequate and needed to be amplified in order to generate sufficient amounts of "representative DNA" for CGH, and other experimental procedures. Five of these DNAs were originally from biopsies and 4 from cell cultures. For whole genome DNA amplification we used the method of multiple displacement amplification (MDA) (Dickson et al., 2005).

2-1-4 GENDER AND AGE DISTRIBUTION

There were 8 females, three of them with the diagnosis of AA. Of the 23 males 20 had GBMs. The patients' ages ranged from 31 to 69 years, with the mean of 46.4 years. Only 6 patients were aged over 55 years, four of them females.

2-2 METHODS

2-2-1 METAPHASE CGH

An investigation of the genome-wide copy number alterations for each of the 33 tumours was performed using CGH. Tumours GBM/C1724 was subjected both to reverse hybridisation and to MDA to compare results with those obtained by the

conventional labelling procedure (details follow). This tumour was in addition investigated by the methods of array CGH and MFISH.

2-2-2 CELL CULTURES

Patients with insufficient or with no remaining frozen biopsy tissues but who had frozen cells had their DNA prepared from this source. Other than the consideration for tumour grade, the selection of cell lines for culture was relatively random. However, the prevailing tendency was to select cell-lines of lower passage (p) times, commonly p2 – p6 although several cultures were of higher passage. For example, GBM/C1724, used for array CGH and MFISH, was of passage number 12.

2-2-3 RE-ESTABLISHING CELL CULTURES FROM FROZEN ALIQUOTS

Aliquots of one milliliter (1 ml) comprising a suspension of ~10^6 astrocytes in a mixture of fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO) which serves as a cryopreservative protecting cells from structural damage during deep freezing in liquid nitrogen at temperatures of ~ minus 196C, were recovered from liquid nitrogen, thawed at room temperature and transferred into a 25ml sterile universal container where cells were washed in 9mls Hank's balanced salts solution (HBSS). The cells were then pelleted from solution by centrifugation at 1000 rpm for 5 minutes and the pellet of cells resuspended in 10 mls of the Nutrient Mixture F-10 (HAM), which contained 25 mm Hepes Buffer, fetal calf serum (FCS), L-Glutamine, penicillin and streptomycin (GPS) and NaHCO3 (Invitrogen/Gibco, Paisley, Scotland). The cell suspension in the nutrient mixture was transferred to a 75 cm^3 culture flask and volume made up to 20ml with additional nutrient mixture. Cell culture flasks from Bibby Sterilin (European distributor for the Iwaki range of cell biology plastic-ware) were used for cell cultures. These flasks have a special surface treatment to ensure optimum attachment of anchorage-dependent cells. The flasks were incubated in a chamber of 5% CO_2, at 37°C throughout the period of cell growth. All procedures involving exposure of the cells to the outside were carried out in the safety of the laminar airflow cabinets (Gelaire, INC Biochemicals, Basingstoke, Hampshire).

2-2-3-1 MAINTAINING CELLS IN CULTURE

Cells were inspected on average two to three times each week. This enables early detection and disposal of the occasional infected cultures and feeding the cultures with rapid doubling times. On average cells were fed once a week or when the nutrients were depleted from the medium depending on which came first. When the medium is depleted of nutrients and the amount of metabolic waste predominates, the medium becomes acidic and assumes an increasing bright yellow and pale colour. A flask due for replenishment of nutrients was transferred to the laminar airflow cabinet from where the old media was aspirated and replaced with equal amount of HAM's F10 (Sigma-Aldrich).

2-2-3-2 PASSAGING OF CELL CULTURES

In order to obtain sufficient cell numbers for a high yield of DNA (averaging 10 – $20X10^9$ cells) cells were harvested and passaged three times. Cultures were initially established in one medium (25 ml) flask which, when confluent, was passaged into three flasks of the same size. In the second passage, one of the three was in turn passaged into one large (75 ml) flask while the two remaining were maintained as backup in case of contamination. Subsequently, they were frozen if there was no immediate need. Once confluent, the large flask was passaged into three flasks of the same size. The passage process involved aspirating or draining the nutrient media from confluent monolayer, flask rinsed with 1.0 ml trypsin EDTA (Life Technologies), which was drained and the cells incubated in 3-5 mls of trypsin for 2-3 minutes or until the cells are fully detached. Caution was taken not to over expose the cells to trypsin treatment for fear of inducing damage to the cell membrane. Cells were assisted to detach by intermittently striking the lower edge of the flask against the palm of one hand and gently swirling contents. When a majority of cells were detached and freely floating about, an equal amount of nutrient medium containing fetal calf serum, which naturally contains the enzyme antitrypsin, was added to stop the digestive activity of trypsin. The cell suspension was then pipetted into a 25ml universal container (collecting tube) and cells pelleted by centrifugation at 1000 rpm for 5 minutes. The liquid phase was aspirated and the cells resuspended in 10 mls of HAM's F-10 nutrient medium. This suspension was divided equally between the required culture flasks. The nutrient medium in each flask was adjusted to 20.0mls and 30.0 mls respectively for the

medium and large flasks. The number of times the cell line was passaged was updated from the previous passage number by a factor of one.

2-2-3-2-1 PROCEDURES FOR CONFLUENT CELL CULTURES

When the cells in the large flasks are confluent they are detached with trypsin as previously described. To the trypsin - cell suspension is added 5 mls PBS buffer and cells pelleted by centrifugation at 3000 rpm for 5 minutes. The cells are washed a second time by resuspending in 10 mls PBS buffer from which the cells are pelleted and the media discarded before the cells are resuspended in 10 mls of fresh PBS and the cell counted. A cell suspension containing approximately $2x10^7$ cells is pelleted and resuspended in 2.0 mls PBS buffer and DNA extracted following the QIAgen protocol for DNA extraction from cell cultures. Any surplus cells from back up flasks and in excess of the $2x10^7$ needed for DNA extraction are frozen by resuspending the detached cells in 10.0 mls of nutrient mixture and the cell count determined. The cells are pelleted and resuspended in an appropriate amount of FCS containing 10% DMSO (Sigma Aldrich) to a final concentration of $1x10^6$ cells per ml. Aliquots of 1.0 ml cell suspension are stored in appropriately labelled cryotube vials which are frozen down progressively first to -70^oC for the first 24 hours after which the vials are considered safe for transfer to LN_2 tanks for long-term storage.

2-2-3-3 EXTRACTION OF TUMOUR DNA FROM CELL CULTURES

Genomic DNA was extracted from LN_2 frozen-biopsies and short-term cell cultures using respectively the QIAGEN tissue protocols [DNA Mini Kit] for solid (biopsy) tissues, and for cultured cells (QIAamp DNA Blood Mini Kit Handbook, January 1999) (QIAGEN Ltd, Crawley, Sussex). After elution DNA was stored at -20^oC until needed. DNA purified by the DNA Mini Kits ranges in size up to 50 Kb, with fragments of approximately 20-30 kb predominating. This is an optimal size for preparation of the probe required for CGH by Nick translation and, in addition, DNA of this length is denatured completely during thermal cycling and can be amplified with the highest efficiency.

2-2-3-4 MEASUMENT OF DNA CONCENTRATIONS

After DNA extraction the assay of DNA concentration was done with the DyNAQuant 200 fluorometer (Amersham Pharmacia Biotech, Little Chalfont, Burkinghamshire) using the DyNAquant Capillary Cuvette kit. The DyNAQuant 200 fluorometer is a filter fluorescence photometer for accurate measurement of low DNA concentrations using Hoechst 33258 dye (Sigma, St. Louis, USA for Amersham Pharmacia Biotech). The DyNAQuant 130 kit includes a capillary cuvette and capillary tubes which hold up to 9 µl of solution.

2-3 MULTIPLE DISPLACEMENT AMPLIFICATION (MDA)

The MDA-protocol used in this experiment required the concentration of DNA to be approximately ~10ng/ul. This would yield 5ng of DNA in 0.5ul, which was the initial volume that went into the reaction tube. Other reagents were added to the tube to make up 50ul reaction volume, in the following order: 26.5ul ddH_2O, 12.5ul of dNTP mix and 0.5ul DNA polymerase (table 2-2).

DNA sample ID	**Starting Conc. (ng/ul)**	**Dilution Ratio**	**Post-dilution Conc.**	**DNA Conc. 0.5ul**	**Reaction Buffer** (ul)	**ddH_2O (ul)**	**dNTP mix (ul)**	**Ø29 (ul)**	**Total Reaction Vol (ul)**
AS2721	93	1:9	9.3	4.7	10	26.5	12.5	0.5	50
GBC1724	670	1:99	6.7	3.4	10	26.5	12.5	0.5	50
EF	665	1:99	6.65	3.4	10	26.5	12.5	0.5	50
MPE	112	1:9	11.2	5.6	10	26.5	12.5	0.5	50
Control	10	-	-	5	10	26.5	12.5	0.5	50

Table 2-2 shows the work-up of some of the DNA samples used in this study. *Shaded boxes indicate components that constitute the total reaction mixture for each sample.*

Into each of the tubes containing 0.5ul aliquots of each sample (in the above example, ranging in concentration from 3.4-5.6 ng of DNA) were added 10ul of reaction buffer, and the tubes were then vortexed briefly, spun down, and samples denatured at 95C for 3 minutes, before returning to ice buckets for addition of the remaining reagents. Each tube of denatured-DNA-samples/buffer mix was made up with 26.5 µl of ddH_2O, 12.5ul of dNTPs and 0.5ul Phi29 DNA-polymerase (with reagents added in that order), and immediately transferred to a circulating waterbath, which regulated at 30C, and left to

incubate for 16-18 hours (overnight) (alternatively a thermocycler may be used). After the incubation period tubes were heated at 65C for 10 minutes, to stop the reactions, and samples were then stored at -20C until required. DNA concentration was quantitated with Picogreen Fluorimeter (Invitrogen) following which approximately 1ug of the reaction mix was purified by phenol/chloroform isopropanol precipitation. The pellet was resuspended in 5 µl of Elution Buffer (Qiagen) and a small sample (containing ~50ng) was run on 1% agarose gel to determine the size of DNA fragments.

2-4 LABELLING OF TUMOUR DNA

Tumour DNA was labelled using SpectrumGreen-dUTP (Vysis, Downers Grove, IL). The reagents were mixed in chilled sterile 0.5 ml eppendorf (PCR) tubes, which were wrapped in aluminum foil to protect the reagents from direct light and kept in buckets of ice. Initially, approximately 1 ug of genomic DNA from each tumour was aliquoted into two separate eppendorf tubes and mixed with 2.5µl of 0.2 mM SpectrumGreen, 5µl 0.1 mM dTTP, 10 ml 0.1 dNTP mix (dATP, dCTP, dGTP), 5µl 10 X nick translation buffer. Then 5µl and 10µl nick translation enzyme was introduced into either of the tubes, respectively. The final volume was adjusted to 50µl with nuclease-free water. The tubes containing the mixtures were vortexed briefly and incubated at 15°C for 2 hours. The reaction was stopped by heating at 70°C for 10 minutes in the thermocycler and the samples transferred to ice or stored in refrigerator at -20°C while the probe size was being determined, or until the needed for hybridisation. The optimum probe size for CGH analysis is in the range of 300-3000 bp. As the quality of starting DNA varies greatly between different samples, two volumes of enzyme were routinely used for each sample during the first labelling reaction to ensure that the appropriate sized smear was obtained. After optimizing the labelling protocol we routinely labelled DNA in only one tube, usually digesting 1ug of DNA for 2 hours with 10uL of nick translation enzyme in a 50uL reaction mix, which resulted in an optimum probe size whose length ranged between 500 bp and 3 Kb.

2-4-1 DETERMINIG THE PROBE SIZE

The size of the labelled DNA was estimated by running 50 – 100 ng of the reaction mix from each tube, mixed with 2µl PCR loading buffer on a 1% agarose gel (dissolved in tris-acetate-EDTA buffer (TAE), stained in ethidium bromide (0.5 mg/ml) to aid with

the visualization of DNA under UV light. The 1 KB marker DNA, EcoRI 911 was used to provide the size ladder for estimating the size of the probe. The electrophoresis was carried out with the gel immersed in 1 % TAE, at 100V for 60 minutes; at other times, 60V, or alternatively 50 ma, for 60 minutes, which produced similar outcomes.

2-4-2 PREPARING THE PROBEMIX

CGH was performed according to Mohapatra (1998) with some modifications. For standard CGH experiments, test DNA and normal DNA was labelled by nick translation using DNA *Taq* polymerase (Life Technologies) with SpectrumGreen and SpectrumRed respectively. In order to produce a hybridisation signal with equivalent intensities various amounts of test and normal DNA were hybridised in a ratio of 2:1 SpectrumGreen to SpectrumRed labelled DNA. For most experiments, four hundred nanograms ((400ng) of SpectrumGreen tumour probe was combined with 200 ng of SpectrumRed total genomic reference DNA (Vysis) and 10 mg Cot-1 human DNA (Vysis) in 0.5 ml eppendorf tubes. To each tube was added 0.1 volumes of 3M sodium acetate and 2.5 volumes of 100% ice-cold ethanol to precipitate the DNA. However, at other times 1ug/0.5ug of test to normal DNA with 20 ng of human Cot-1 were ethanol-pelleted in 0.5ml eppendorf tubes using 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice-cold (-20°C) 100% Et OH.

Samples were vortexed briefly and placed at -20°C for 1 hour or in dry ice or –75°C for 15 minutes, followed by centrifugation at 13,000 rpm for 30 minutes to pellet the DNA. After the supernatant was removed the and the sides of the tube blotted dry pellet was dried for less than 1 minute under vacuum and was then resuspended in 10μl of hybridisation mixture, and dissolved in CGH hybridiation buffer containing 50% formamide, 10% dextran sulfate. The probe mixture was denatured in a water bath at 73°C for 5 minutes immediately prior to hybridisation, and hybridised to normal metaphase spreads (Vysis Inc., Downers Grove, IL) for 72 hours. Control experiments using Vysis-labelled SpectrumGreen and SpectrumRed normal DNAs (for negative controls) and Vysis-labelled MPE600 (for positive controls) were performed at each hybridisation experiment.

2-4-3 HYBRIDISATION TO METAPHASE SPREADS

Normal metaphase slides made from phytohaemaglutin-stimulated lymphocytes derived from a karyotypically normal male donor were procured from Vysis. The factory process involved culturing lymphocytes for 48-72 hours prior to synchronisation with thymidine to produce chromosomes with lengths of 400-550 bands. Hybridisation areas were marked with a diamond pen and the identity of the tumour indicated at the label end of the slides using a sharp pointed lead pencil. The metaphases were denatured for 5 minutes at 73°C by immersing in 50ml jar of denaturing solution containing formamide, 20xSSC and nuclease-free water, then dehydrated serially by immersion for 3 minutes in ice-cold 70% ethyl alcohol, followed by 2 minutes' duration each in 85%, and 100% ethanol.

While the slides were drying, the probe was similarly denatured for 5 minutes at 73°C waterbath, following which the tubes containing the probe were kept in a warm environment (waterbath at 50°C) until time of hybridisation. After air-drying the metaphase slides, 10 μl of the denatured probemix from two different tumours was applied to previously designated hybridisation sites on the normal metaphases slides, which were immediately covered with coverslip and sealed with cow gum prior to incubation in a sealed (+/- humidified) container for 72 hours, at 37°C.

2-4-4 POST-HYBRIDISATION WASHES AND COUNTERSTAINING WITH DAPI

The cow gum seal and coverslip were removed on the third day. Slides incubated in a dry chamber needed immersion in cool room temperature (RT) 0.4xSSC/0.3% NP-40 to loosen the gum so that the slide covers can be removed. Otherwise, the slides are normally washed by immersion in warmed 0.4x SSC/0.3% NP-40 at 74°C for 2 minutes, which is followed by immersion in 2x SSC/0.1% NP-40 at room temperature for 1 minute, prior to air-drying in darkness. Hybridisation sites on the dry slides were counterstained with 4,6-diamidino-phenylindole (DAPI) in antifade solution (Vysis).

2-4-5 DIGITAL IMAGING AND ANALYSIS

Metaphase images with uniform hybridisation were acquired with a Quantitative Image Processing System (QUIPS) software using a Zeiss Axioscope epifluorescent

microscope equipped with a triple band pass filter (Vysis) designed to simultaneously excite and emit light specific for DAPI, SpectrumGreen and SpectrumRed, and a cooled charge-coupled device camera (Photometrics, Tuscon, AZ). The hybridisations were evaluated by visual inspection in the course of image acquisition, and during digital image analysis. A hybridisation of good quality is easier to karyotype and results in accurate interpretations of the data within and across experiments. Metaphases were selected for CGH analyses if they had minimum or negligible background fluorescence, (that which was low and uniform around each chromosome) with minimal surrounding cytoplasm and balanced red and green fluorescence. It is essential that the chromosomes are well developed, with an average length of 400 – 550 band, adequately separated from each other and should preferably be free from overlapping. It is preferable to have all the chromosomes of a metaphase accessible in the X100 magnification microscope-objective so as to capture all of them in a single digital image during acquisition.

2-4-6 CAPTURING METAPHASE IMAGES

Chromosome images from hybridised metaphases were captured using SmartCapture softwares, and the features of this program are described briefly here. The SmartCapture program has several features for capturing multicolour images of metaphases resulting from hybridisation. The camera settings are configured to enable the condenser on the microscope to give the best illumination and duration of exposure to light. A binning of 2 x 2 is usually set for a 100x objective. Once the capture settings have been verified, the rest of the capture procedure is automated through a series of dialogue boxes, which allow the operator to decide whether to save or discard images that have been acquired. Suitable images are exported and saved in an ICS format (described below). When the file is saved in the ICS format, two files are automatically created; one ".ics", the other, an ".ids." The ".ics" file contains a text description of the image while the ".ids" file contains the image data. Both of these files were required to analyse the image using the Quips program.

2-4-7 EDITING THE METAPHASE AND KARYOTYPE

The process of editing the metaphase karyotype was done in the main window, which is displayed on the computer screen. The CGH/Karyyotyper program provides for multiple metaphase image analysis to generate a single karyotype. Each window also

displays a selection of command options and tools used to edit the metaphase, such as tools for separating a touching pair, overlapping or cluster of chromosomes, and it also allows the operator to include/exclude unsuitable chromosomes from a karyotype or from a CGH profile, as well as repositioning those that are inverted and for classifying or declassifying chromosomes.

2-4-8 CLASSIFIYNG CHROMOSOMES

There are several methods for classifying chromosomes using the Quips software. The software will automatically classify the chromosomes by size, using the banding-specific classifier. Generally, there will be one or more errors in the computer-generated karyotype, and the operator then manually edits the results to complete the karyotype. The chromosomes are organized in a standard human karyotype format to facilitate examination. Several features are available for evaluating and editing a karyotype, for example, the "Reference window", which is used for examining a metaphase image in the original form. To facilitate a review of the classification, several features of the chromosome can be selected for the computer to display automatically, such as the centromere and axis of symmetry, or an option can be selected to show an ideogram and how to align the chromosomes. A set of tools is included that can be used to reposition a centromere, rotate and straighten chromosomes and exclude objects from the karyotype. A good knowledge of distinguishing characteristics and normal alignment of the entire chromosome complement is necessary for the operator to be able to recognize, identify, separate and match closely resembling chromosome sets and groups. On the basis of size, the chromosomes and classes into which they fall are ordered in decreasing length: typically 1 – 7, X, 8 – 22 and Y. The Quips CGH/Karyotyper program has classifiers for use with DAPI-, G- and R-banded chromosomes.

2-4-9 EXAMING A CGH RATIO PROFILE

A ratio profile of the fluorescence intensities is measured along each chromosome. To facilitate review of the profile data, it is possible to select various features, such as ideograms, to display in the main window, or to show the grid lines on the profile and the gain/loss bars. It is possible to exclude ratio profiles from profile analysis.

2-4-10 INTERPRETING CGH PROFILES

The interpretation editor of the Quips Interpreter program is used to select and evaluate the profile data from several metaphases in a single experiment. By combining the ratio profiles of homologous chromosomes from several metaphases, a reliable identification of likely areas of change in the sequence copy number is possible. The Interpreter program calculates the mean ratio and determines the standard deviation, confidence interval, and range for the data.

The ratios of fluorescence intensity along the chromosomes were quantitated as described by Piper et al (1995). The mean ratios of SpectrumGreen to SpectrumRed signal intensities were calculated with SmartCapture software (Vysis). On average between 10 and 20 metaphases were analysed and averaged to yield CGH profiles for each tumour. A relative gain was scored when the mean green:red ratio was above 1.2 and relative loss, when the mean green:red ratio was below 0.8. CNAs were not scored at or near the centromeres. Gains were scored only when visual inspection revealed a bright and discrete signal confined to a subchromosomal region. If signals were too dim, the hybridisation was repeated. Similarly, if ratios were simultaneously altered at chromosomes 1p, 19, and 22, the experiment was repeated as recommended in Mohapatra et al., (1998).

2-5 ARRAY-BASED COMPARATIVE GENOMIC HYBDRID-ISATION (arrayCGH)

Array CGH was performed using the Sanger Centre slides: numbers11174, and 1309-42 – 1309-47 (Sanger Centre Cambridge, UK), which were kindly provided respectively by Professor Ian Tomlinson of Cancer Research UK, and Dr Maria Bitner-Glindzicz of the Institute of Child Health (ICH), Great Ormond Street Hospital for Children, UCL. All 7 were 1Mb genomic arrays. The hybridisation experiments were performed by Dr Rosemary Ekong of the Galton Laboratory, UCL, at the genetics laboratory of the Institute of Child Health (ICH; UCL), where Rosemary was involved in conducting other genetic studies using micro arrays. In addition the ICH has an established platform for microarray analyses.

2-5-1 LABELLING OF TARGET DNA

A total of 10 μg of genomic DNA from test samples and normal controls was first digested with Mbo1, then purified using the QIAquick® PCR purification kit (Qiagen, www.qiagen.com). A total of 2 μg of Mbo1-digested genomic DNA was then labelled by random priming with dCTP-Cy3 or dCTP-Cy5 (Amersham Biosciences, www.amershambiosciences.com). The reaction contained 300 μg/ml random octamers (BioPrime kit, 18094-011; Life Technologies), 0.5 mM dATP, 0.5 mM dTTP, 0.5 m dGTP, 0.2 mM dCTP, 80 U Klenow (Life Technologies, www.lifetech.com or NEB, www.neb.com) and 2 ml dCTP-Cy 3 or dCTP-Cy5 in a total reaction volume of 50 μl. Purifications were on Centri-Sep columns (Princeton Separations, www.prinsep.com) and storage was at – 20°C. Estimates of the amount of purified labelled DNA were obtained from ethidium bromide-stained gels of 1 μl of the Cy5-labelled DNA.

2-5-2 PREHYBRIDISATION AND HYBRIDISATION

Denatured slides were prehybridised with 10 μl of preheated (100°C for 3 min) 50% formamide, 5 x SSPE, 0.5% SDS, 5 x Denhardts, 100 μg/ml herring sperm DNA, 1 μg/ml human COT1 DNA, and an ethanol-cleaned 22x22-mm glass coverslip was placed over the arrayed area. The slide and coverslip assembly was placed in a humid box for 30 min at 37°C. Cy3- and Cy5-labelled test and reference DNA (3–4 mg of each) were combined in a microcentrifuge tube and spin dried under vacuum at room temperature, then suspended in 10 ml of hybridisation buffer (50% formamide, 5 x SSPE, 0.5% SDS, 60–80 μg human COT-1 DNA). This mix was denatured at 100°C for 3 min and the DNA was preannealed at 37°C for 30 min. For hybridisation, the mix was placed on a new coverslip, which was overturned onto the slide to allow the DNA-hyb mixture to spread evenly before the coverslip was gently slid off to remove as much prehybridisation mix as possible without scratching the array. The slide was inverted so that the array side was facing down and the hybridisation mix on the new coverslip was immediately picked up and allowed to spread over the array area. The slide/coverslip assembly was placed in a humidified sealed Perspex slide chamber and incubated at 42°C for 16 hr.

2-5-3 POSTHYBRIDISATION

Washes were performed at 45°C using 50 ml prewarmed solutions and gentle agitation. Coverslips were floated off with 2 x SSC/0.1% SDS. Washes were 4 x8 min in 50% Formamide/2 x SSC/0.1% SDS, 4 x5 min in 2 x SSC/0.1% SDS, 1 x5 min in 0.2 x SSC/0.1% SDS, 1 x5 min in 0.02 x SSC/0.1% SDS, and a final rinse in sterile distilled water. Slides were dried by centrifugation at 1000 rpm for 3 min then stored in the dark.

2-5-4 SCANNING AND NORMALIZATION

The arrays contained ~3000 clones, covering the entire genome, most of them in duplicate although a small number were in quadruplicate sets. A rapid scan (40 mm resolution) of the microarray was performed with the Cyclone Gene Array Scanner (ScanArray®) using QuantArray image analysis software (Perkin Elmer: www://las.perkinelmer.com/). An area judged to be representative of the maximum signal was rescanned at higher resolution (10 mm) to minimize exposure of the microarray to the scanner's lasers thus avoid significant photobleaching of the dyes. The software can save the image analysis data in a tab-delimited text file and automatically export the file to an Excel spreadsheet for spot-by-spot examination of the data.

2-5-5 ANALYSIS OF ARRAY DATA

Despite experimental caution, variations occur within and between experiments. These variations, which result from random and systematic errors, affect measurements and, in turn, decrease accuracy. While random errors can be minimized through replicate measurements, systematic errors (biases) need to be controlled experimentally and require statistical correction (Nadon and Shoemaker, 2002). Systematic errors, associated with the properties of the different dyes, spotting, labelling strategy, hybridisation and posthybridisation conditions, quality of DNA samples, and scanner settings during data collection, result in variations (Nadon & Shoemaker, 2002; Quackenbush, 2002; Yang et al., 2002). Those biases associated with dyes include differing rates of incorporation of the different dyes during labelling, signal intensity of the different dye complexes, differing background noise, and the physical properties such as half-life and light sensitivity (Ekong et al., 2004). Therefore, before comparing

the copy number of DNA sequences, data needs to be transformed so that 1) measurements due to artifacts are removed, 2) measured intensities are adjusted, and 3) the relative ratios (copy numbers) of the genomic sequences determined. Normalization is the initial transformation applied to microarray data, and balancing the data sets by normalizing with a set of appropriate control samples should minimize variations (Nadon & Shoemaker, 2002; Yang et al., 2002). Due to the presence of systematic variations in array experiments, each slide was normalized separately, using a set of controls (Ekong et al., 2004). Data acquired from the Cyclone Gene Array Scanner were analyzed with the QuantArray image analysis software (Perkin Elmer). For each spot, the software gave values for the red signal, the green signal, the local background, and the signal intensity after subtraction of the local background. Results were presented as ratios of red:green. In addition, the software generates whole chromosome and individual chromosome plots. In the array experiments no matching blood or normal DNA were available for comparative analysis with the patients' tumour samples.

2-6 MFISH

2-6-1 TISSUE CULTURE AND HARVESTING

Passage 12 (p12) short-term culture of GBC1724 was established as described in section 2-2-3. When the cultures were >70% confluent (average 48-72 hours) colchicine (Colcemid, Life Technologies, Gaithersburg, MD, USA) was added into culture and left for 40 minutes to synchronise the cells in metaphase. The cells were then harvested, washed, and resuspended in 0.075M KCL (hypotonic buffer) at 37C for 15 minutes. A small volume of 3:1 methanol:acetic acid fixative was added to the cell suspension and 3 more fixative washes were subsequently performed. Cell pellet(s) were stored at -20C in fixative, until required for metaphase spreads preparation.

2-6-2 SLIDE PREPARATION

The equipment required for this step included a waterbath in which the water temperature was maintained at 75-80C. Although any waterbath would work, a waterbath with re-circulating capabilities close to the water surface was preferable, since the amount of steam produced is higher. The water level was brought to within 1-2 cm from the top to allow heating of a flat metal surface (the lid of any metal plate) close to the temperature of the water. The metal plate was placed on top of the

waterbath, covering it only partially, leaving an opening of about 5-10 cm through which hot steam could escape. At the other end of the metal plate, an area ~10 cm wide was allowed to project ("hang out") in the air and not be in contact with the steam. A 2-3mm thick metal plate was used, which allowed a smooth temperature gradient across the metal plate surface, from ~70C at the "hot" end (facing the water) to about room temperature at the "cold" end of the plate.

2-6-2-1 MOUNTING METAPHASES ONTO SLIDES

Using a common dropper, or an automatic pipette, 20-25 ul cell suspension was placed on a slide taken directly from its original package. Dropping from a height was not necessary. The suspension was spread evenly by moving the pipette tip gently across the slide surface. Excess liquid was drained on a paper towel. When the surface of the slide became grainy (as the fixative evaporated), the slide was placed facedown for 1-3 seconds in the line of steam escaping from a hot waterbath, then it was dried by placing on the metal plate carrying a gradient of temperature across its surface, as described already. The hot steam is intended to overcome any potential influence of the atmospheric humidity, while the drying-temperature was varied depending on the positions on the metal plate where slides were placed to dry out.

Dried-out metaphase-mounted slides were examined, to determine the quality of the metaphase spreads, under the x10 or x20 objective (lens) of an Olympus microscope equipped with a charged coupled device (CCD) camera (Carl Zeiss, Welwyn Garden City, UK).

2-6-2-2 CHEMICAL AGEING OF SLIDES

Ethanol was used to achieve fixation and shape-preservation of the nuclei/chromosomes on the slide. The slide was place on the metal block of a thermocycler and 150-200 µl of ethanol pipetted onto the slide, which was then covered with a coverslip. The slide/coverslip assembly was in turn covered with ethanol-soaked gauze to prevent ethanol evaporation. The thermocycler block was programmed to increase its temperature to 94C, and to hold it there for 10-20 seconds before gradually cooling down towards room temperature. Depending on the machine, the heating and cooling speed is 1-2C/second.

An alternative method was to incubate the slide for 10-15 seconds each, in jars with ethanol at 50C, 75C, 94C, 75C and 50C, followed by drying at room temperature. Gradual thermal treatment works better than sudden temperature changes for chromosome shape preservation. To harden the chromosomes more and ensure sharper subsequent DAPI banding, the time the slides are incubated at 94C in ethanol can be increased up to 2 minutes.

2-6-2-3 PEPSIN TREATMENT OF METAPHASE MOUNTED SLIDES

Slides chemically aged for 10-15 seconds at 94C were incubated in 20-30ul of a 10% (w/v) solution of pepsin in HCL (0.5ml) made up with 49.5 ml distilled water, for 30-60 seconds, rinsed briefly in PBS and incubated for 5 minutes each in 70% and 100% ethanol, followed by air-drying. Slides that were aged longer (i.e., for 2 minutes at 94C) were subjected to 1-2 minute pepsin pretreatment.

2-6-3 SLIDES & DNA-PROBE DENATURATION & HYBRIDISATION

Slides were treated with 150ul of 70%formamide/2xSSC and cover with a 50x22mm coverslip, place slide on the metal block of the thermocycler and denature as described under chemical ageing. Once the thermocycler has cooled down to safe temperatures, slides are removed, the coverslip taken off and slides place for 3 minutes duration, each time, in 70% and 100% ethanol at room temperature, and then air-dried.

The MFISH chromosome probe (SpectraVysion, Vysis, Richmond, Surrey, UK) was suspended in 50ul of formamide hybridisation buffer and denatured by heating the vial for 5 minutes in a thermocycler at 75C and then cooling down to room temperature. The probe was left to re-anneal for ~ 30 minutes, then it was placed on the warmed slide, covered with coverslip and sealed with rubber cement and incubated for ~24 hrs (overnight) in a moist chamber at 37C.

2-6-4 POST-HYBRIDISATION WASHES AND DETECTION

Slides are washing serially in Detergent Mix solution made from 2:1 parts of 4XSSC and Detergent DT solutions, respectively, followed by further washes in Stringency was

solution, made from equal volumes of 1xSSC and Deionised formamide (50 mls of each). The solutions, placed in Coplin jars (i.e., Stringency wash solution (2 jars), 1xSSC (2 jars), and Detergent wash solution (1 jar)) are prewarmed to 45C in a waterbath for at least 30 minutes before starting. Slides are removed from the incubation chamber and left in 1XSSC for 5 minutes. The rubber cement is then taken off and slides left in the solution to remove the coverslip. The stringency wash is then done twice, each time incubating slides for 5 minutes, in separate jars of Stringency wash solution. In the final wash, the slides are incubated in the Detergent wash solution for 4 minutes. Taking precautions not to allow the slide to dry, 125ul of Detection Reagent, constituted by mixing Detection Reagent (MDA) and Detergent Wash Solution in a ratio of 1:9, was applied onto the slide and covered with paraffin immediately. Slides were then incubated in a humidified chamber for 15-20 minutes, paraffin removed and slides washed 3 times, for 4 minutes each, in Detergent wash solution at room temperature by emptying and refilling the Coplin jar. The slides were then drained well, and mounted with 50ul of DAPI II®

The MFISH slides were viewed using a fluorescent microscope fitted with epifluorescence filters specific for Cy3, Cy3.5, Cy5, Cy5.5 and FTIC (Carl Zeiss, Welwyn Garden City, UK), imaged using a CCD camera, and karyotyping was performed using Quips SpectraVysion software (Applied Imaging, Newcastle, UK).

3 CHAPTER 3 RESULTS of METAPHASE CGH ANALYSES

3-1 Sample size

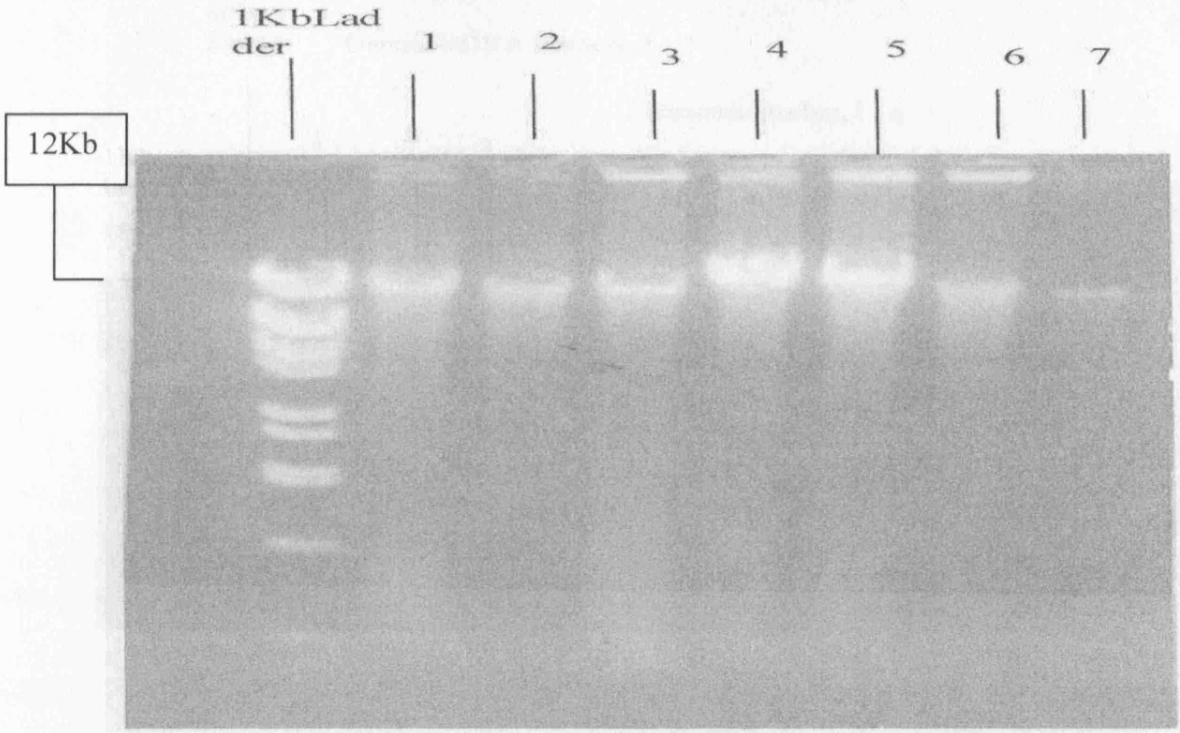
CGH data were obtained from brain tumour samples taken from 32 high-grade astrocytomas, six AAs and 27 GBMs. The patient from whom biopsy number GBC1612 was obtained had tumour recurrence following initial treatment and underwent re-operation, and biopsy RGBC1724 was taken. RGBC1724 was investigated to a greater extent in the course of this study. Its images and other data from various laboratory procedures undertaken throughout the study are presented in Chapter 4 to illustrate outcomes of CGH, MFISH and micro-array CGH procedures.

3-2 Validation of metaphase CGH Procedures

Validation experiments were designed to serve as controls, to validate the outcomes of the Nick-translation steps of the CGH procedure. This is the enzyme digestion of genomic DNA intended to produce smaller-sized DNA fragments that are simultaneously tagged with reporter / fluorescent molecules called the probe. In addition, validation procedures were used to test the quality of probe hybridisation to normal metaphases chromosomes mounted to a microscope slide. Trial experiments and various forms of internal controls were performed at key stages of the experimental procedures. The labelling steps of the CGH procedures were validated through simultaneous but separately labelling (different reaction mixtures/tubes) of test DNA and the unlabelled positive control DNA in the same experiment. This permits assessment of enzyme digestion that is simultaneous with the incorporation of the fluorescent label onto control and test DNAs under similar experimental conditions. The sizes of labelled genomic DNA probes that result from this process were determined by gel electrophoresis (see sample images below). Similarly, the conditions of a subsequent step, the hybridisation experiment, were monitored by simultaneously hybridising factory labelled control DNAs, the negative (normal DNA) or positive (MPE600, a breast cancer cell line with known chromosomal aberrations) controls at the same time as the test tumour probes are being hybridised. Thus the figures that follow illustrate the following sets of sample images,

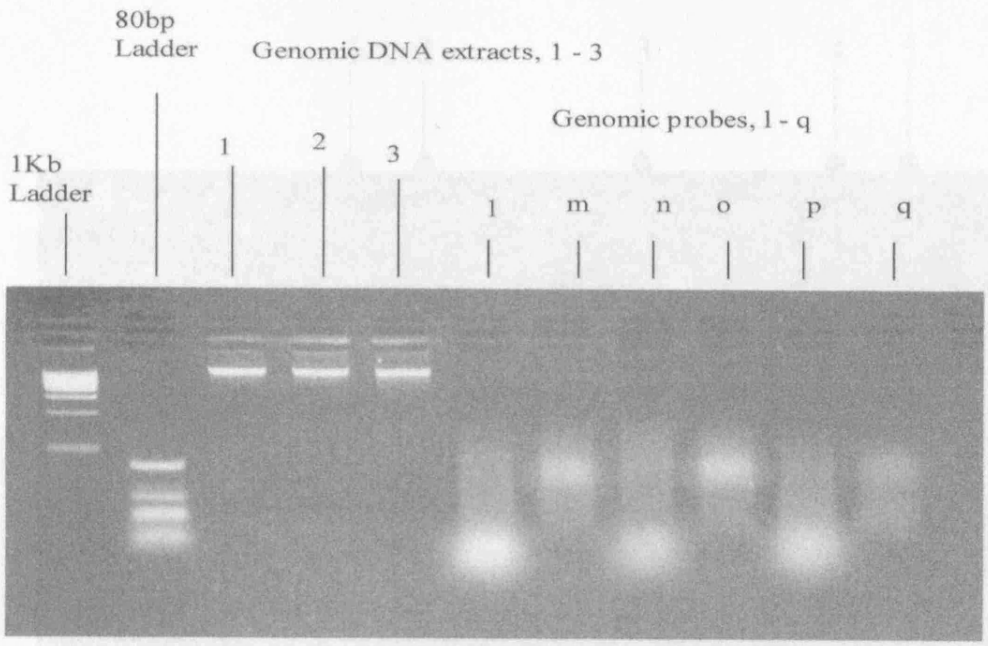
1) Several photographs of 1% agarose gel electrophoreses of DNA extracts and labelled probes. Samples of PGBS1625 (a solid), RGBC1724) (taken from cell culture and MDA-amplified DNA (RGbC1724mda)), originally unlabelled Vysis positive control (MPE600), Galton negative (normal) control (identified as Galton EF) as well as labelled probes thereof are highlighted.

2) A series of CGH images in the form of metaphase spreads, karyotypes and profiles, the results of trial experiments to validate the MDA-amplified DNA for use in metaphase comparative genomic hybridisation. The images are subdivided and arranged in the following order: images of hybridisation of the DNA of RGBC1724 prior to MDA amplification (1) when the CGH experiment was performed with the test probe and reference labelled conventionally, i.e., green and red for test and reference DNAs respectively, (2) the CGH experiment done with the test probe and controls labelled in reverse order, and (3) the CGH experiment performed using the DNA of RGBC1724 that has been subjected to MDA amplification procedure. However, so as to illustrate other aspects of the CGH procedures, such as an initial general assessment of the success of the extraction procedure, and in order to roughly estimate the average size of the fragments making up the DNA extract, sample images of photographs taken following gel electrophoresis of genomic DNA extracts and probes are included.



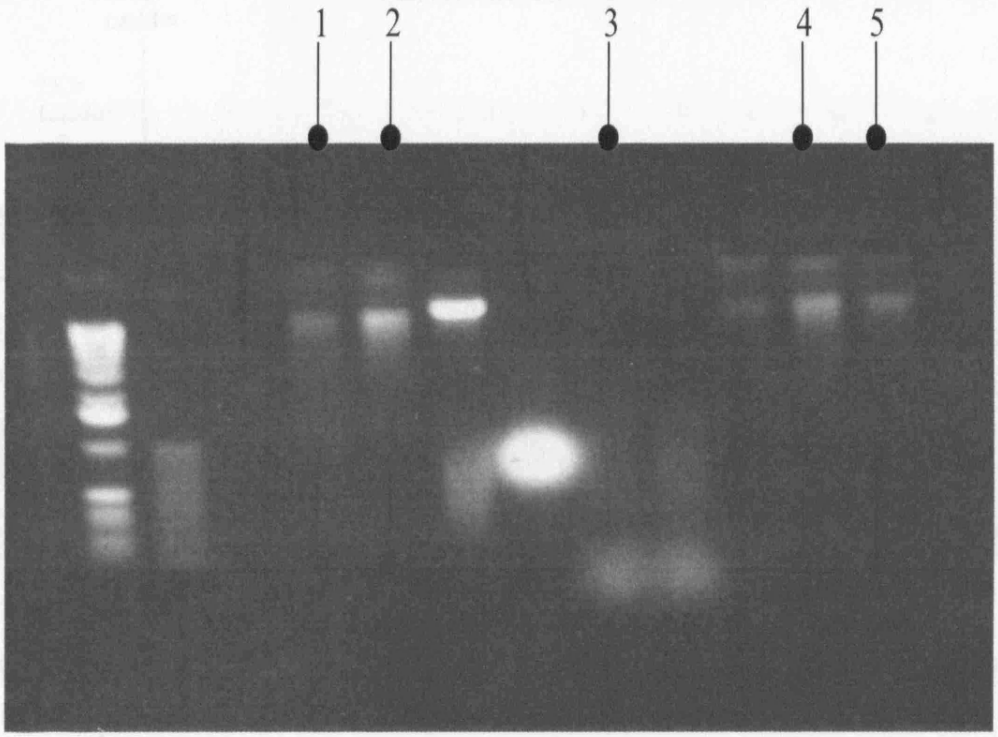
3-2-1 Figure 3-1 Gel photograph of a sample of genomic DNA extracts.

Uncut and unlabelled genomic DNA from tumour samples run in 1% agarose gel and compared with separation of 1 Kb ladder. Lanes: 1=PGBS2051, 2=PGBS2126, 3=AS2614, 4=AS2706, 5=AS2721, and 7=AS2745. All the samples show DNA with fragments sizes around 12 Kb and also showing lack of degradation. The gel was stained with ethidium bromide.



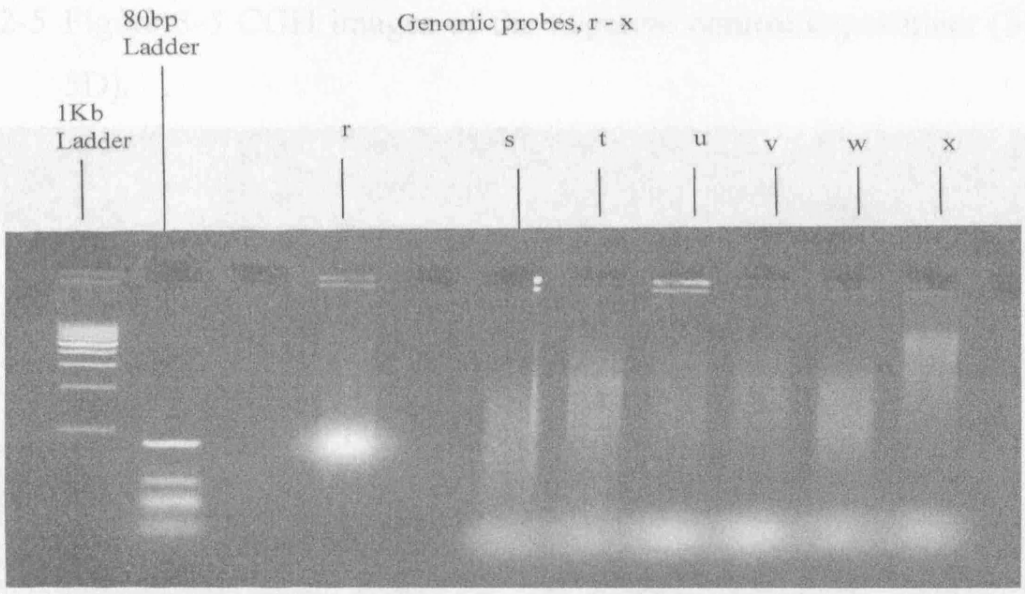
3-2-2 Figure 3-2 Samples of genomic extracts of solids/biopsy tissues.

1% agarose gel stained with ethidium bromide, showing 3 uncut, unlabelled samples 1, 2, and 3 respectively of AS2706 (subsequently excluded from the study), AS2721, and PGBS1625. In lanes l, n, and o are probes made from samples 1, 2 and 3 respectively. All the probes are labelled with Spectrum Green dUTP. The results of the gel electrophoresis of Spectrum Red dUTP labelled samples are shown in lanes m, o, and p. The unincorporated Spectrum Green and Spectrum Red are shown with sharp flare of fluorescence at the ends of lanes l, n, q (unincorporated Spectrum Green dUTP), and in the middle of the DNA smears in lanes m, o, and q (unincorporated Spectrum Red dUTP). Comparison of the smears of labelled probes with the markers suggests fragment sizes in the region of about 200 – 1000 base pairs.



3-2-3 Figure 3-3 - Gel photograph of MDA amplified DNA of RGBC1724 and negative (normal) control DNAs

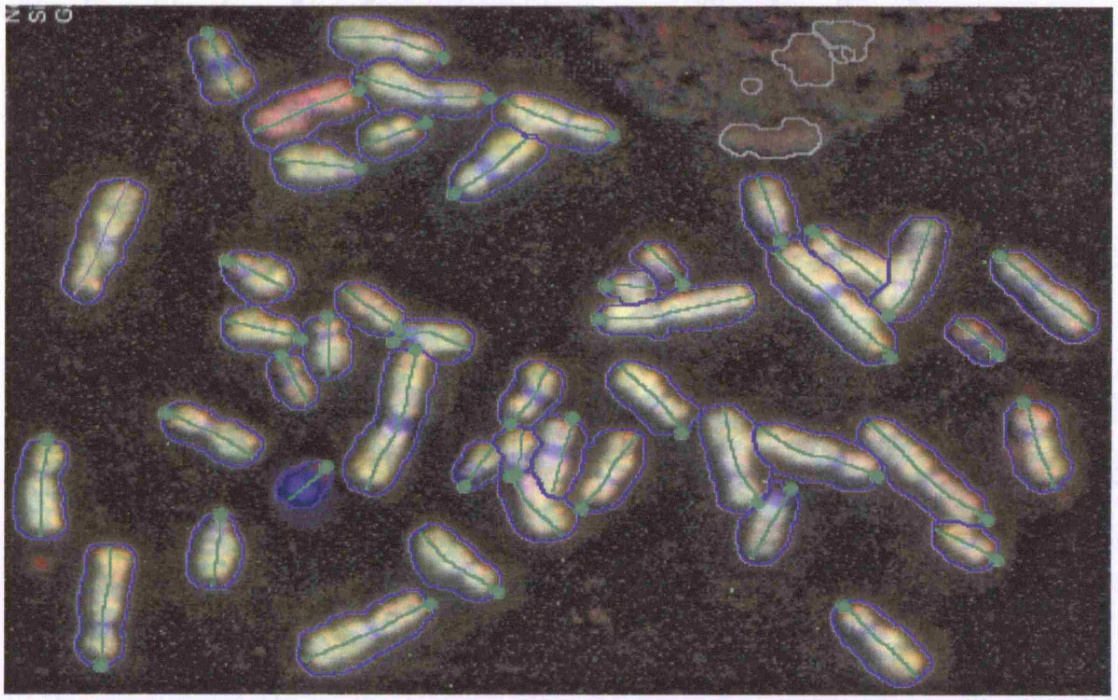
Lanes numbered 1 and 2 are, respectively, those for DNAs of Galton normal (negative) control identified simply as EF, and RGBC1724. These DNAs have been cleaned/purified by phenol/chloroform method and then ethanol pelleted. Lane 3 is of the Spectrum Green-labelled probe of RGBC1724. The MDA-amplified DNAs of EF and RGBC1724 prior to PCI 'cleaning' are in lanes 4 and 5 respectively. The lane immediately before lane 4 contains the control DNA that was supplied with the MDA Kit.



3-2-4 Figure 3-4 Sample genomic probes, r – x.

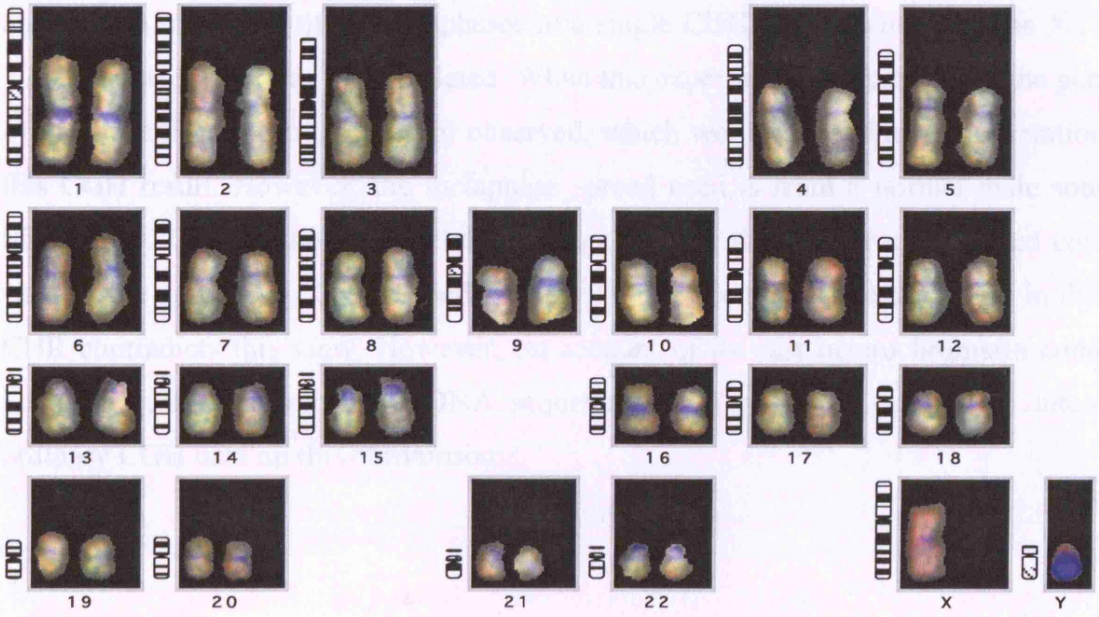
Probe (r) was Galton normal (control) DNA, simply identified as EF. This sample is labelled with Rodamine. Lanes s – x were of probes prepared from genomic DNA, respectively, of GBM/C2227, GBM/S2495, AS2614, GBM/C2410, GBS2867, and GBM/S1830, following MDA amplification. The average probe sizes are in the range of 700-1000Bp, which is somehow over-digested (borderline?). Due to the fact that the starting amounts of DNAs were low, that combined with overdigestion may have contributed to poor hybridisation of most of these tumours. With the exception of AS2614 and GBS2867 the rest were excluded from the study for reason of failure to produce good quality hybridisations.

3-2-5 Figure 3-5 CGH images of the negative control experiment (3-5A – 5D).

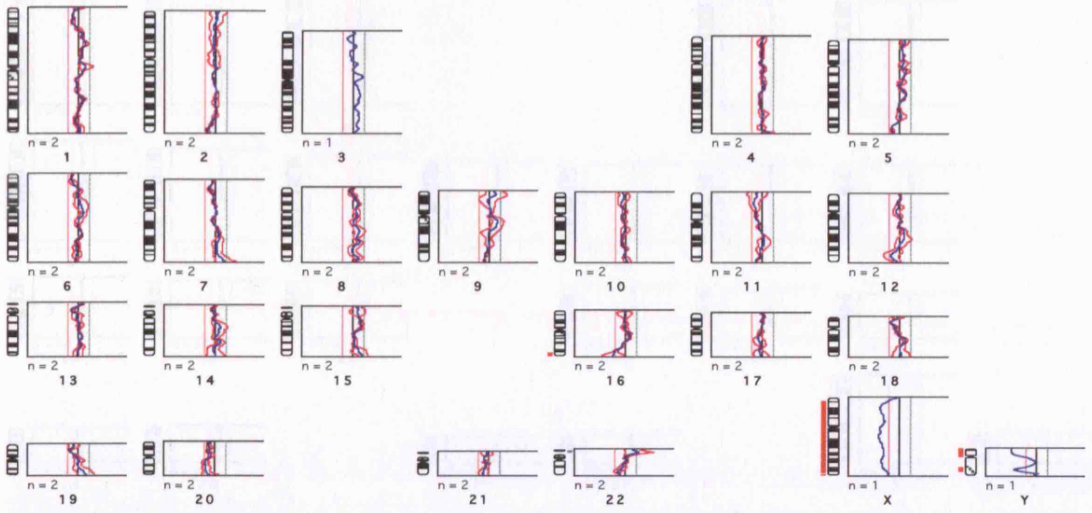


3-2-5-1 Figure (3-5A) Sample metaphase spread of negative control CGH experiment.

The negative control DNAs used were factory-supplied, labelled reference DNAs, one of them labelled green with fluorescein isothiocyanate and the other labelled red with Rodamine.

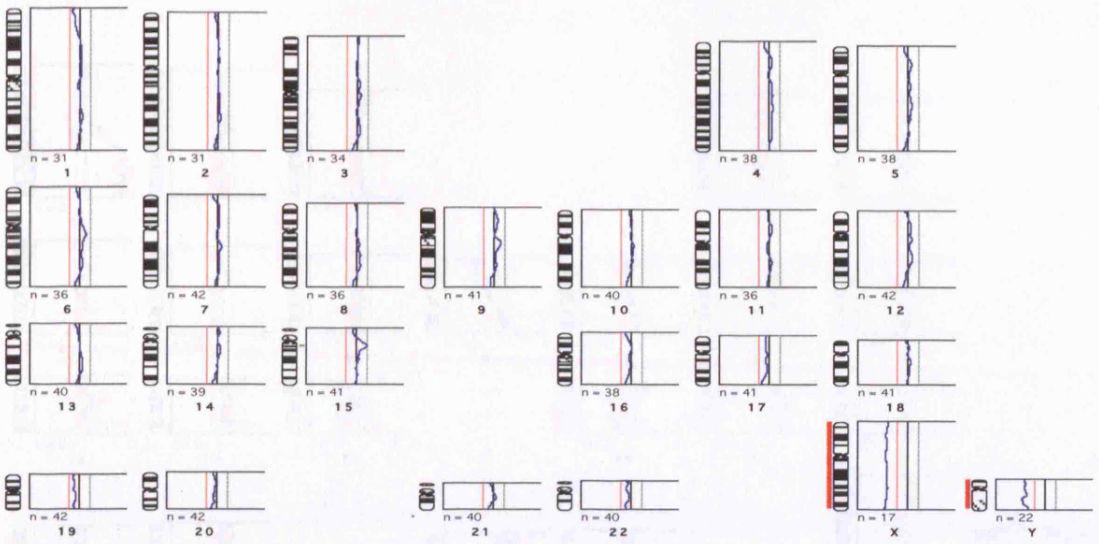


3-2-5-2 Figure (3-5B) Karyotype of the metaphase in (A)



3-2-5-3 Figure (3-5C) Profile of fluorescence ratios for each chromosome-set in the metaphase shown in 3-5A, generated from its karyotype (3-5B).

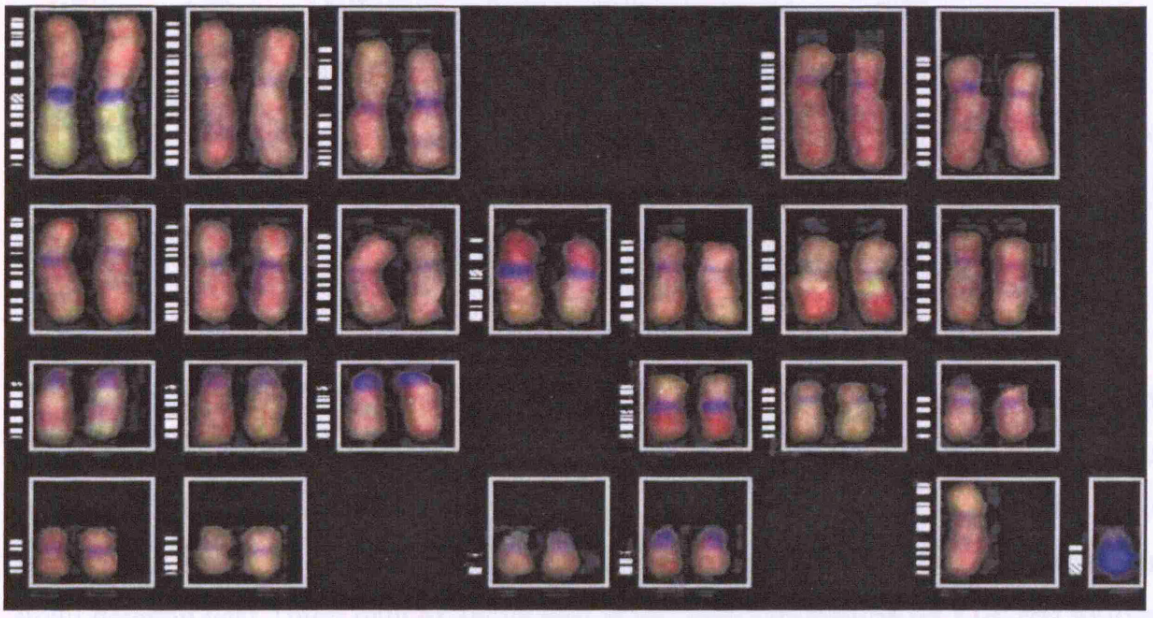
In this single metaphase, the average fluorescence ratio profiles for each pair of the autosomes, except CHR 3 where only one chromosome was available for analysis, are basically normal. But, there is a loss in 16q-ter, which may be due to an experimental artifact. It is partly for this reason that it is necessary, for a reliable outcome to take the average of individual ratio profiles from a minimum 5 chromosomes (Kallioniemi et al., 1995) in a single experiment. In practice most investigators currently recommend analysing a minimum of 10 metaphases in a single CGH experiment. Both the X-, and Y-CHRs are depicted as being deleted. When this experiment was performed the gender of the control DNAs used was not observed, which would have aided interpretation of this CGH result. However, the metaphase spread used is from a normal male source. Since the X-CHR is shown as deleted, it suggests that the Rhodamine labelled control was female and the Sp Green labelled control was male. The concurrent loss in the Y-CHR contradicts this view. However, on account of its vast heterochromatin content, i.e., rich content of repetitive DNA sequences, it is generally difficult to interpret ordinary CGH data on this chromosome.



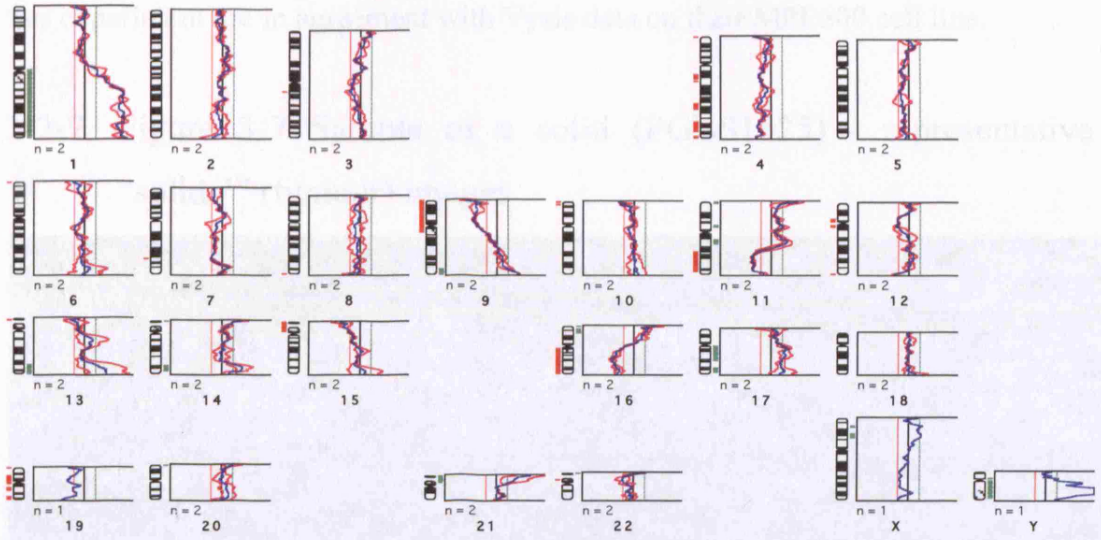
3-2-5-4 Figure (3-5D) Combined ratio profile of 22 metaphases analysed in the negative control experiment.

This figure shows the combined fluorescence profiles for 22 metaphases, with an average of roughly 40 chromosomes anaylsed for each autosome. Note that, unlike in the single metaphase seen earlier, the ratio profiles are now within normal range. The alterations seen in the sex chromosomes, which has been discussed is confirmed.

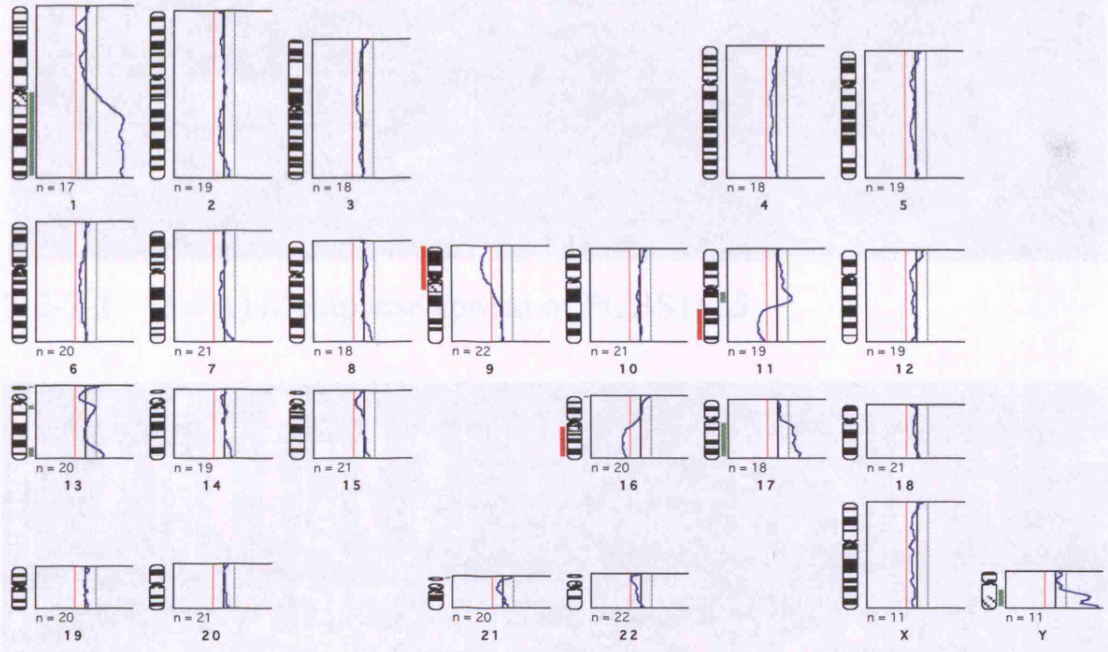
3-2-6 Figure 3-6 - (6A – 6C): CGH images of the positive control (MPE600 (Vysis) v Ref. (Vysis).



3-2-6-1 (3:6A) Karyotype set of a metaphase captured from the positive control hybridisation



3-2-6-2 (3-6B) Profiles of fluorescence ratios of a single metaphase



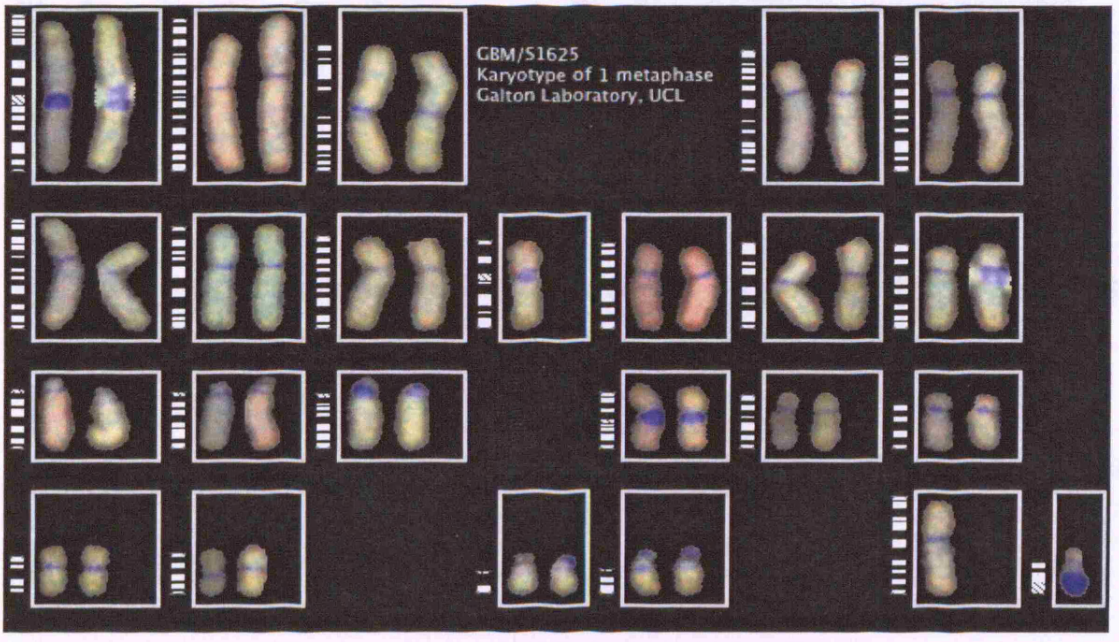
3-2-6-3 (3-6C) Combined ratio profile of 11 metaphases analysed

The alterations revealed included gains at 1q, proximal 11q, distal 13q, and 17q; and deletions at 9p, 11q, and 16q. Most of these agree with results provided by Vysis (the supplier of the control DNA). According to Vysis, the cell line may change over time and could show additional aberrations. However, the cell line is expected to show 1) a small deletion near 1pter; gain of 1q, 2) loss of 9p, 3) distal deletion on 11q, and 4) loss

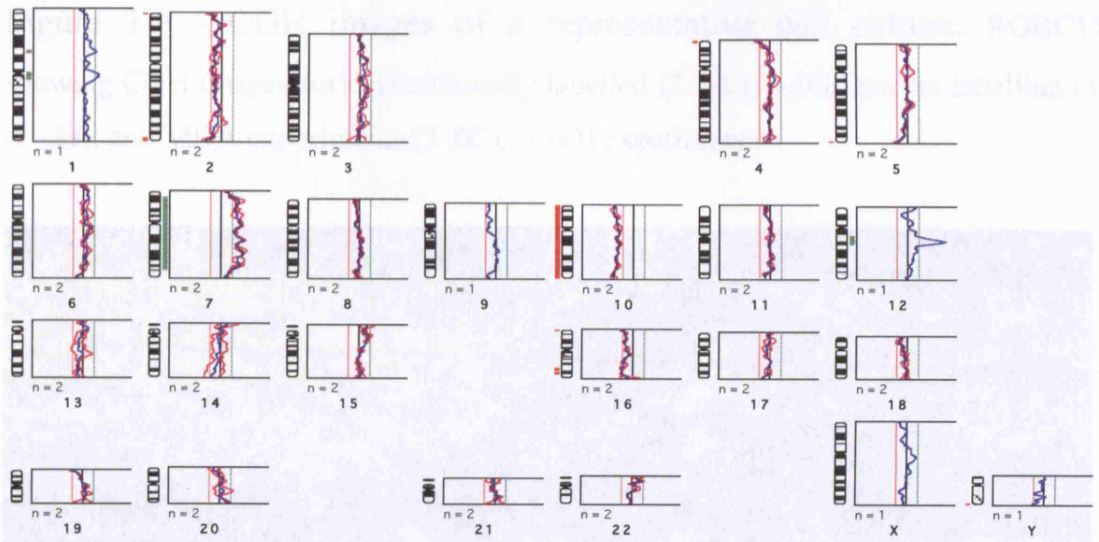
of 16q. Chromosomes 2 and X are expected to be normal. Thus, cytogenetic findings in this experiment are in agreement with Vysis data on their MPE600 cell line.

3-2-7 Figure 3-7 Sample of a solid (PGBS1625) - representative of "solids'" (tumour) images

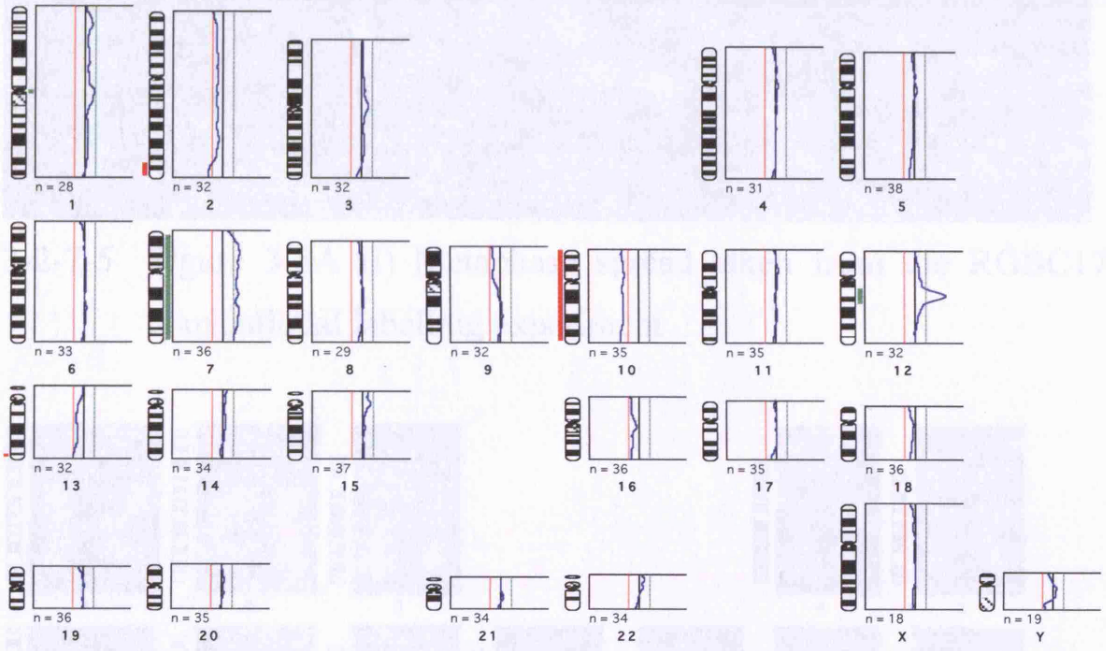
3-2-7-1 (3-7A) Metaphase spread of PGBS1625



3-2-7-2 (3-7B) Karyotype of the metaphase in 3A



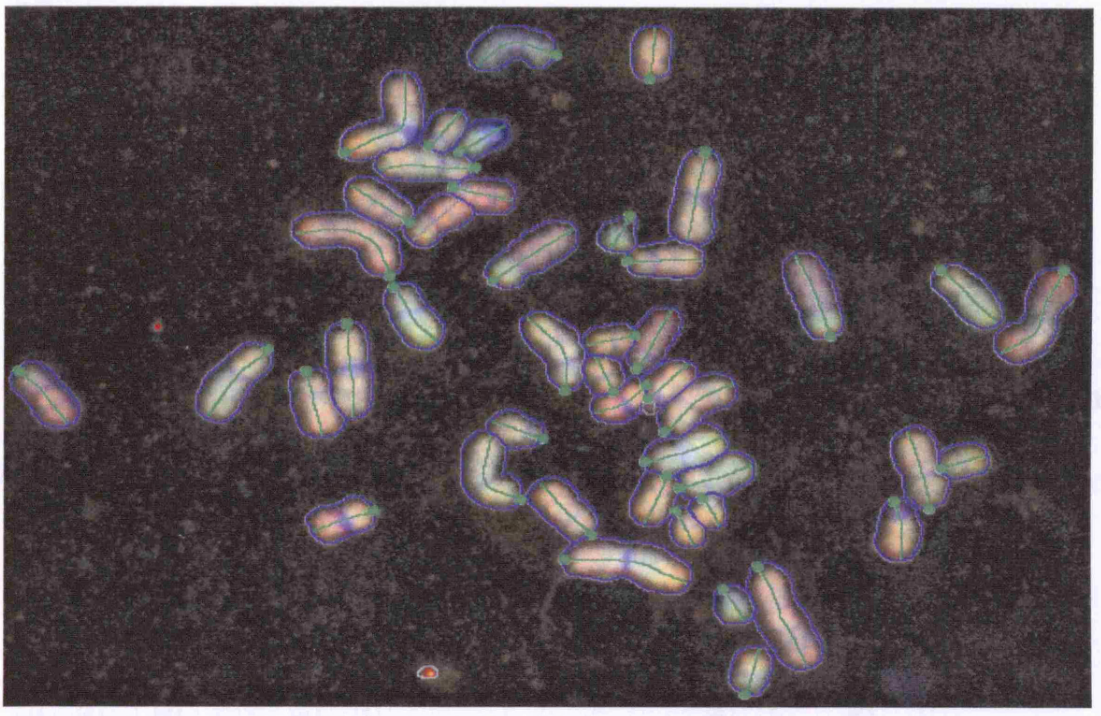
3-2-7-3 (3-7C) Fluorescence ratio profiles of the metaphase in 3-7A, generated from its karyotype (3-7B)



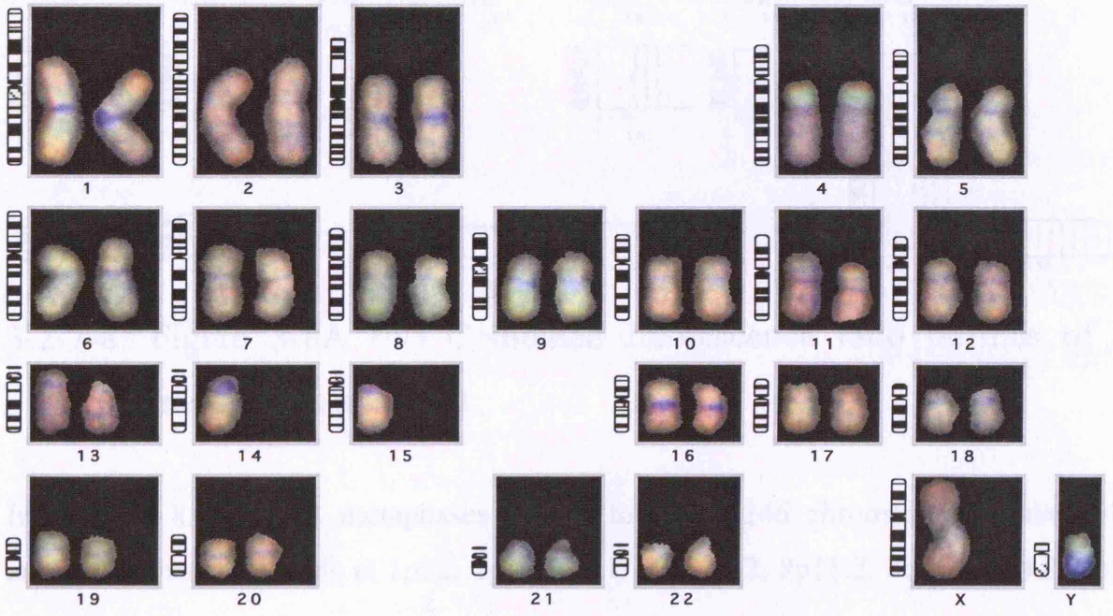
3-2-7-4 (3-7D) Combined fluorescence ratio profiles of 19 metaphases analysed for PGBS1625 in this experiment.

Gains (Green) were observed at 1p11-q11, 7p22-q36, and 12q13-q15, while losses occurred at 2q35-q37, 10p15-q24, and 13q33-q34

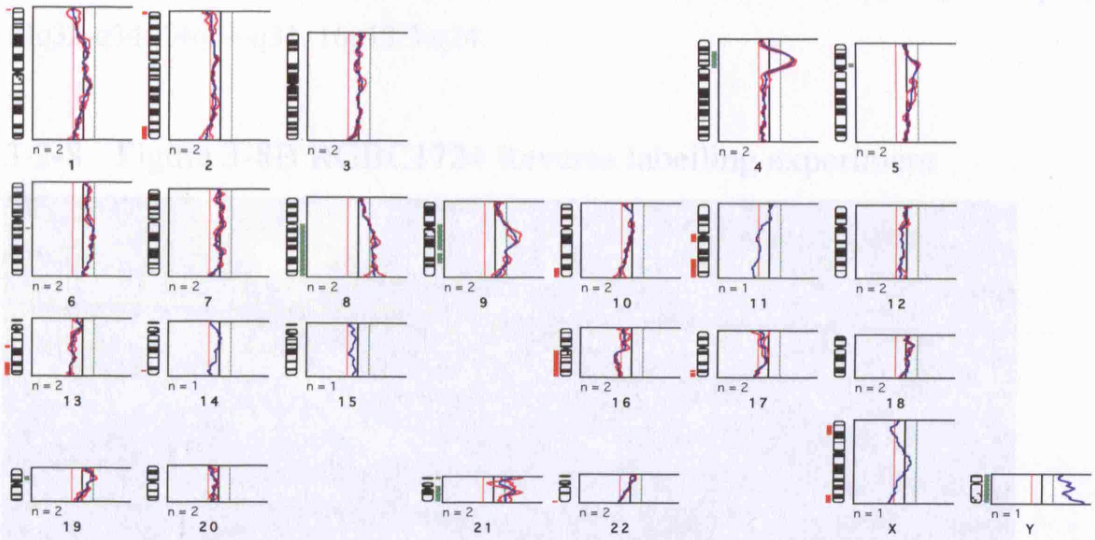
Figure 3-8 - CGH images of a representative cell culture: RGBC1724, showing CGH images for conventionally labelled (3-8A (i – iv), inverse labelling (3-8B (i – iv), and MDA experiments (3-8C (i – iv)) experiments



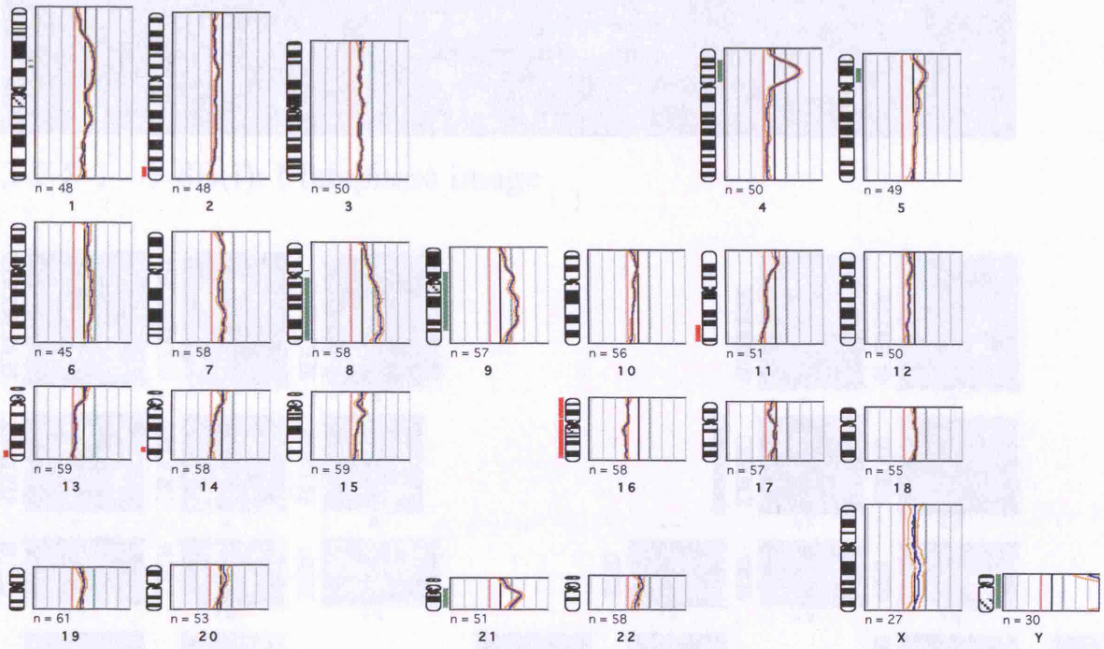
3-2-7-5 Figure 3-8A (i) Metaphase spread taken from the RGBC1724 conventional labelling experiment



3-2-7-6 Figure 3-8A (ii) Karyotype of the metaphase in 3-8A (i)



3-2-7-7 Figure 3-8A (iii) Fluorescence ratio profiles of a single metaphase, same one in 3-8A (i)

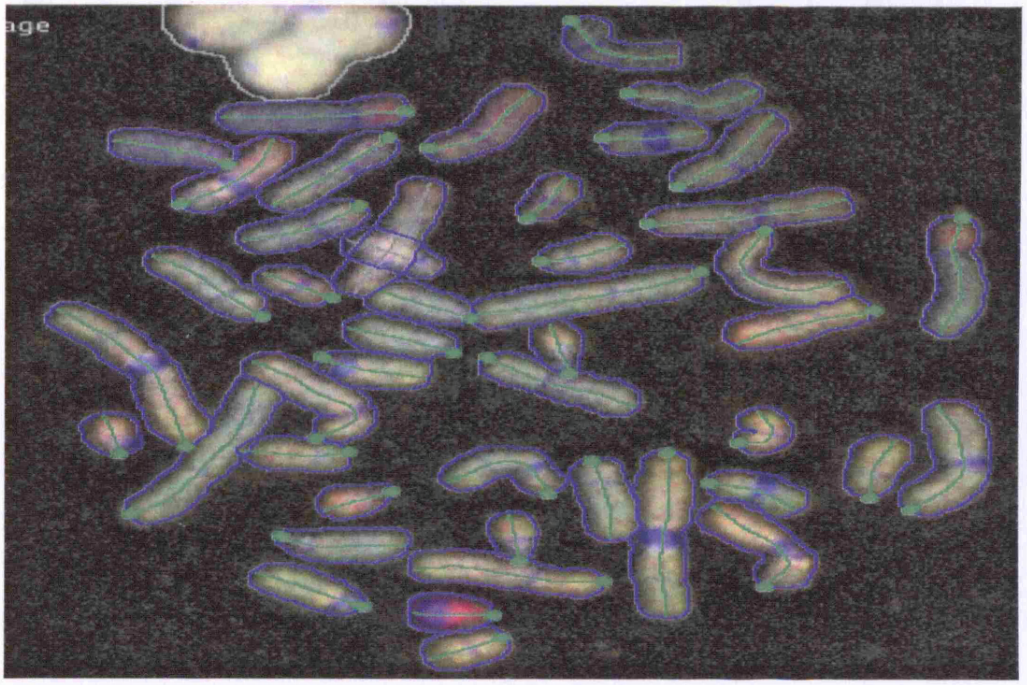


3-2-7-8 Figure 3-8A (iv) Combined fluorescence ratio profiles of 33 metaphases analysed

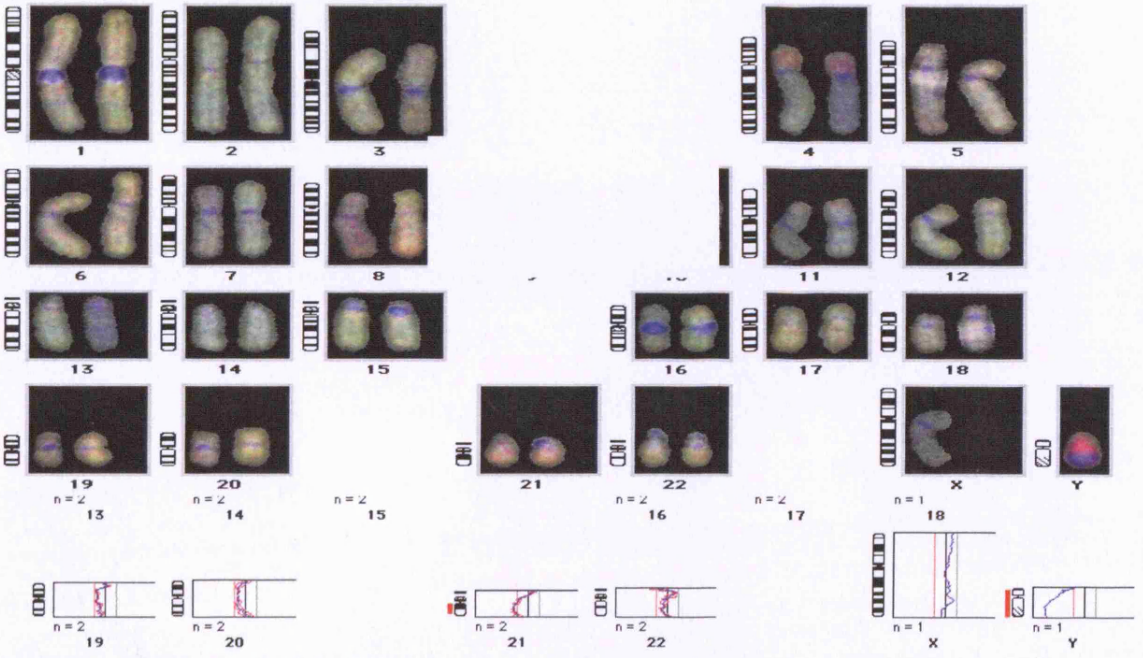
In figure 3-8A (iv) 33 metaphases with a total of 1246 chromosomes have being analysed. There are gains at 1p22, 4p15.3-p11, 5p14-p12, 8p11.2, 8q11.1-q24.3, 9p12-

q33, 21q11-q22, and Yp13.3-Yq12, while losses are observed at 2q35-q37, 11q22-q25, 13q32-q34, 14q24-q31, 16p13.3-q24.

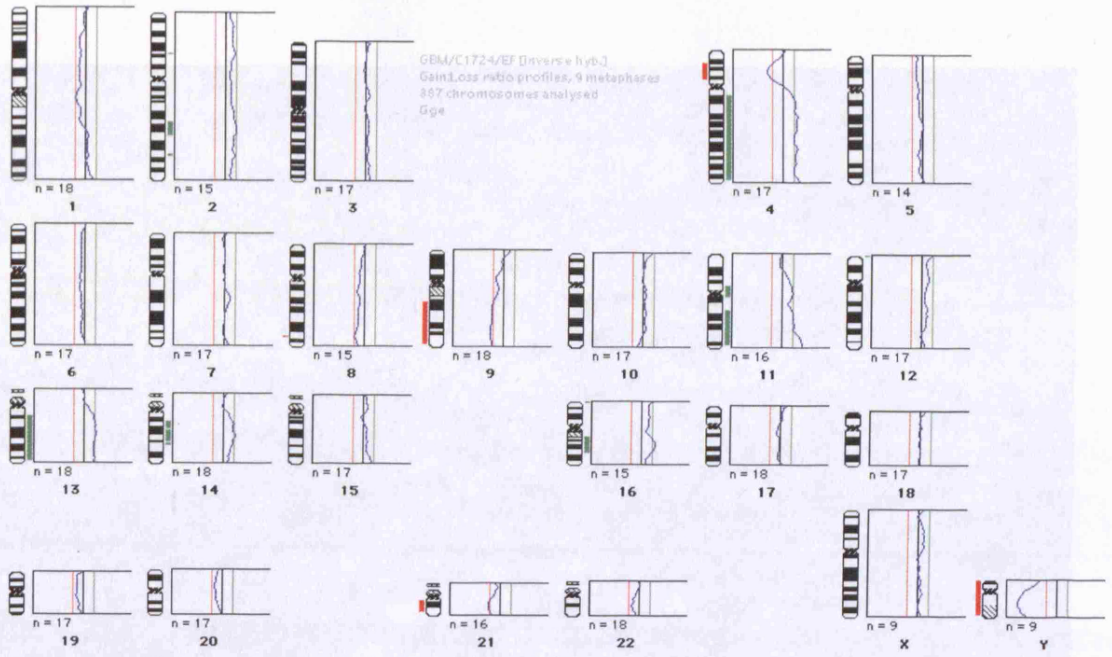
3-2-8 Figure 3-8B RGBC1724 Reverse labelling experiment



3-2-8-1 3-8B(i): Metaphase image

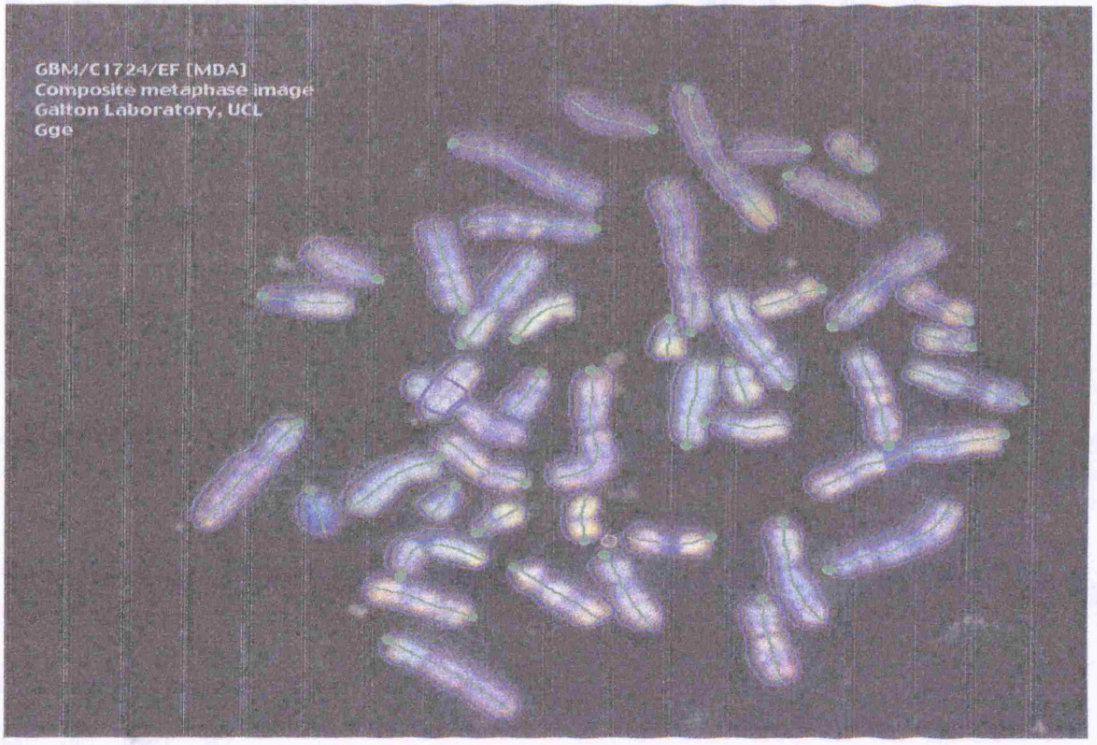


3-2-8-3 Figure 3-8B (iii) Profile of the metaphase in 3-8B (i)

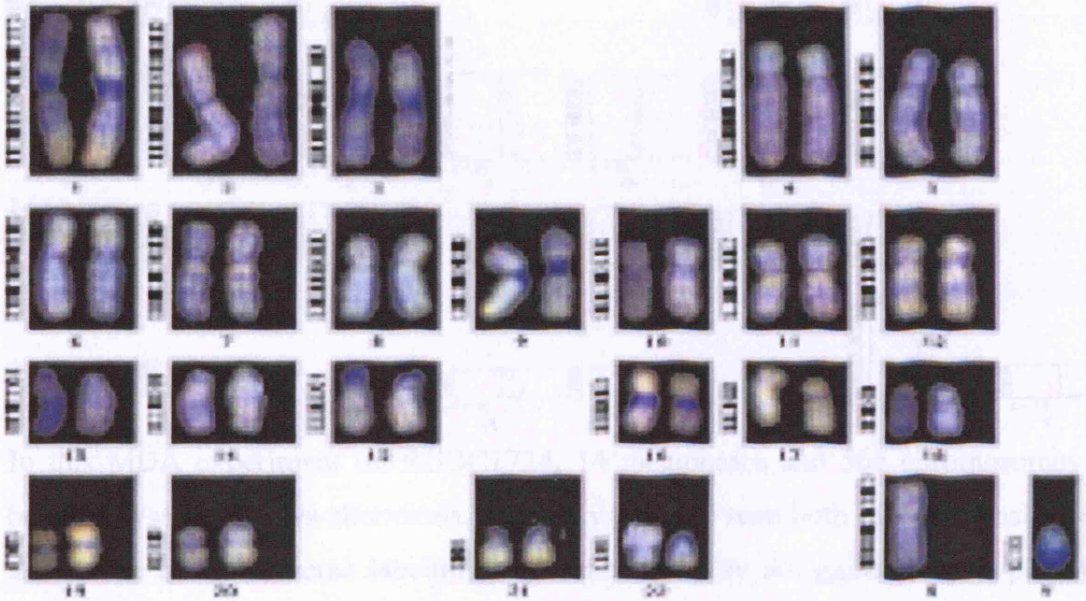


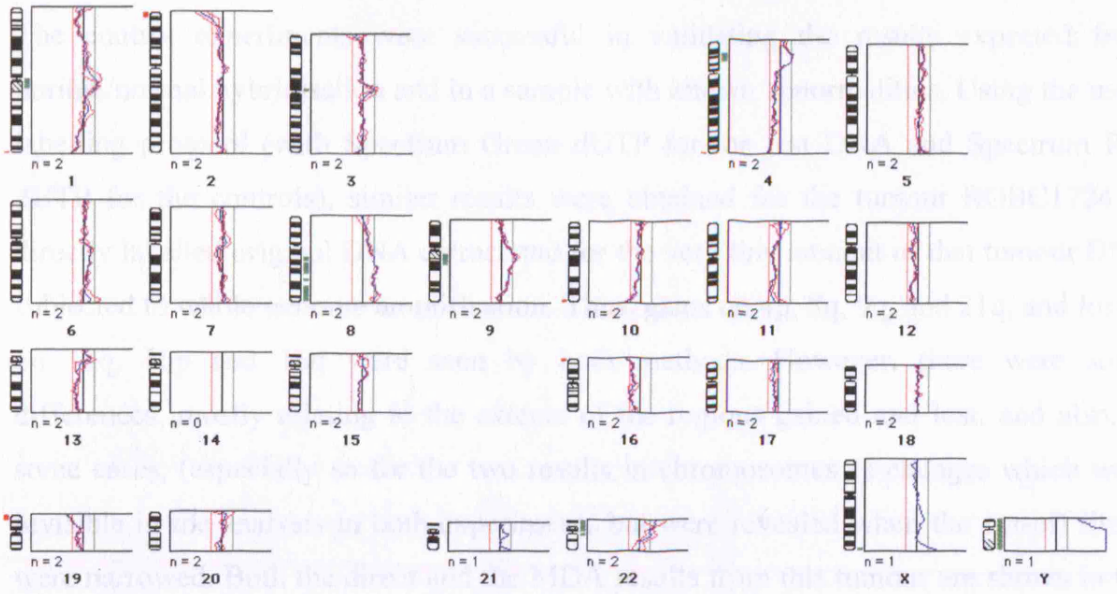
3-2-8-4 Figure 3-8B (iv) Combined fluorescence ratio profiles for The reverse labelling experiment of RGBC1724

Figure 3-8C CGH images of RGBC1724 using MDA-amplified DNA

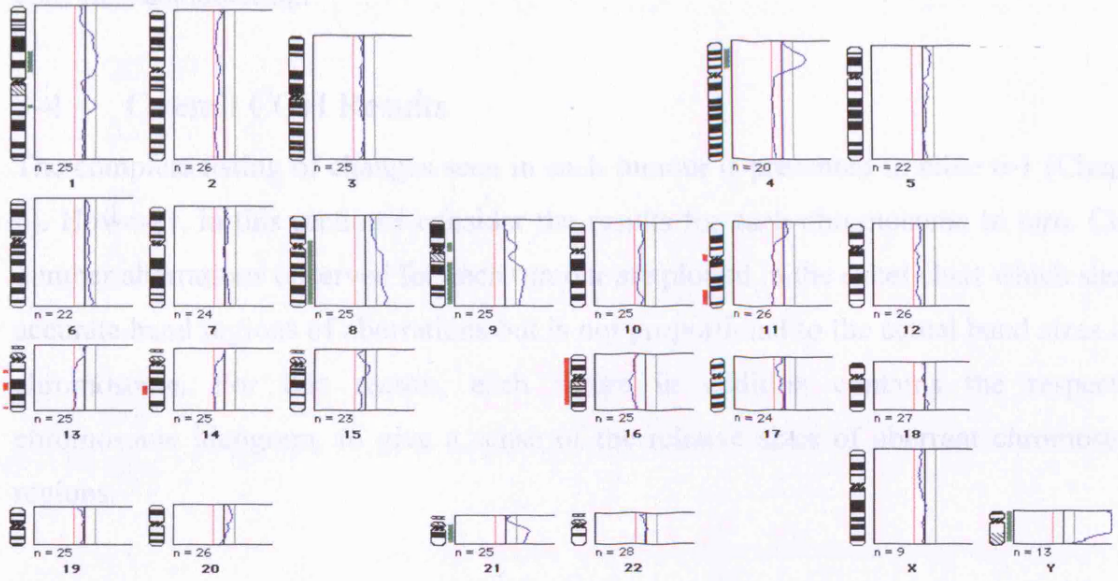


3-2-8-5 Figure 3-8C (i) Metaphase image





3-2-8-7 Figure 3-8C (iii) Fluorescence ratio profiles for a single metaphase



14

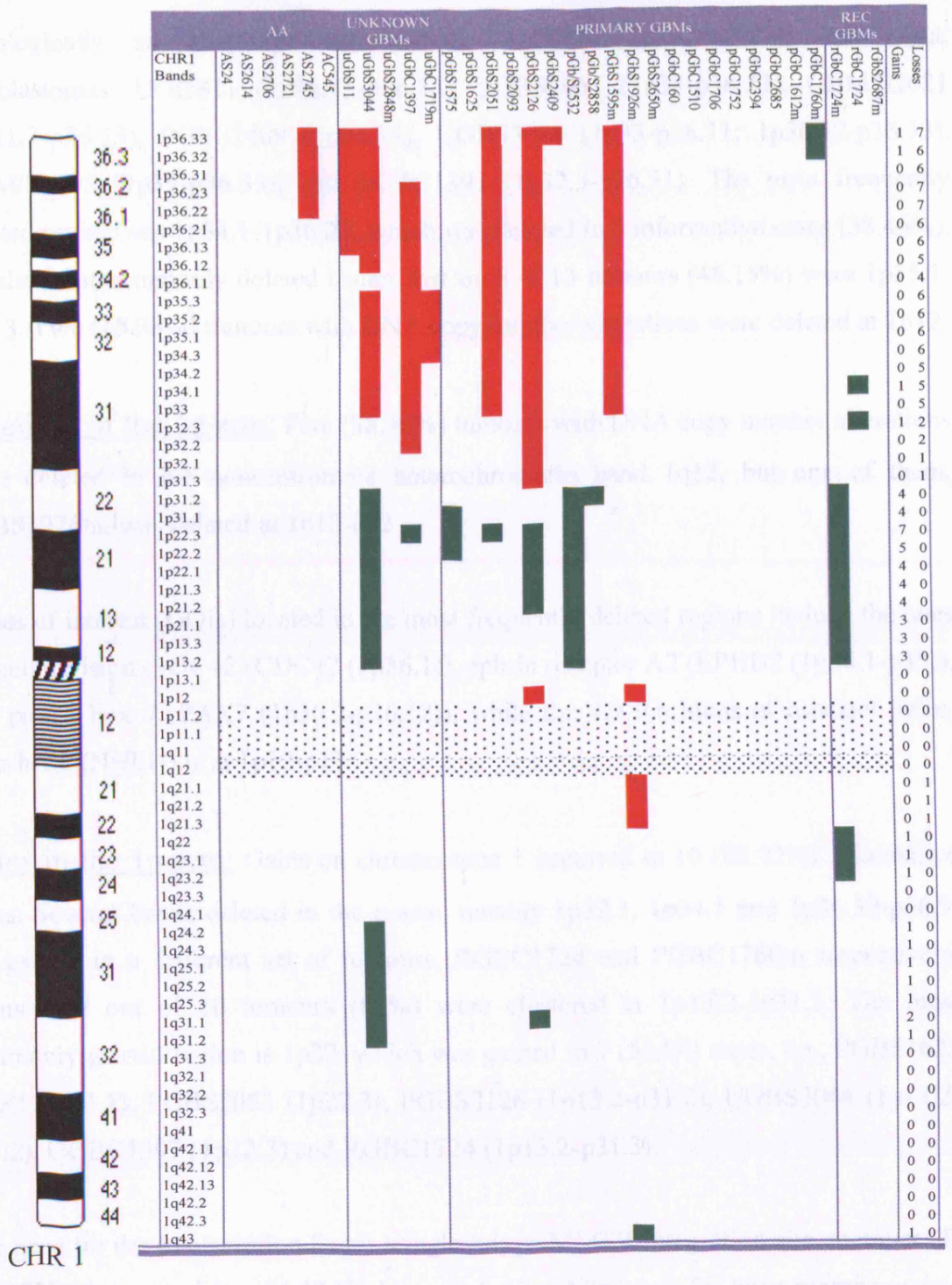
In this MDA experiment on RGBC1724, 14 metaphases and 566 chromosomes have been analysed. It shows alterations, most of which are seen both in the normal labelling and in the inverse/reverse labelling experiments. There are gains at 1p31-p13, 1q22-q23, 4p15.3-p12, 8p11.2-q24.3, 9p13-q11, 9q13-q34, 21q11.2-q22, and Yp11.3-q22; and losses at 11p11.2-q11, 11q22-q25, 13q13-q14, 13q33-q34, 14q21-q24, and 16p13.3-q23

3-3 Conclusions From Control Experiments

The control experiments were successful in validating the results expected from normal/normal hybridisation and in a sample with known abnormalities. Using the usual labelling protocol (with Spectrum Green dUTP for the test DNA and Spectrum Red dUTP for the controls), similar results were obtained for the tumour RGBC1724 as directly labelled original DNA extract and for the very tiny amount of that tumour DNA subjected to whole genome amplification. Thus, gains on 4p, 8q, 9q, and 21q, and losses on 11q, 16p and 16q were seen by both methods. However, there were some differences, mostly relating to the extents of the regions gained and lost, and also, in some cases, (especially so for the two results in chromosomes 1) changes which were invisible in the analysis in both experiments but were revealed when the cut-off limits were narrowed. Both the direct and the MDA results from this tumour are shown in the subsequent analysis, emphasizing the somewhat arbitrary nature of the cutoff values, which have been used. The reverse labelling experiment proved less sensitive although the major changes were still seen. All subsequent experiments were carried out with the conventional labelling.

3-4 Overall CGH Results

The complete listing of changes seen in each tumour is presented in table 6-1 (Chapter 6). However, in this section I consider the results for each chromosome in turn. Copy number aberrations observed for each tumour are plotted in the excel chart which shows accurate band regions of aberrations but is not proportional to the actual band sizes in a chromosome. For this reason, each figure in addition contains the respective chromosome ideogram, to give a sense of the relative sizes of aberrant chromosome regions.



3-4-1 Figure 3-9 - Chromosome 1: Ideogram and Excel chart showing CNAs for 16 high-grade astrocytomas (HGAs).

(Losses and gains are represented in red and green respectively).

<u>Deletions in the 1p-arm:</u> Chromosome 1 was deleted in the distal p-arm between 1p31.1 and 1p36.33 in 9 out of 16 (60%) informative cases, of which one (6.7%) was

histologically an anaplastic astrocytoma, AS2745 (1p36.3-p36.3), and eight, glioblastomas: UGBS11 (1p36.13-p36.33), PGBS2051 (1p33-p36.33), GBM/S2621 (1p31.3-p36.33), PGBS2409 (1p36.33), UGBS3044 (1p33-p36.11; 1p36.12-p36.33), GBM/S1595 (1p33-p36.33), and UGBC1397 (1p32.3-p36.31). The most frequently deleted region was 1p34.1-1p36.23, which was deleted in 5 informative cases (38.46%), but the most commonly deleted bands, lost by 6 of 13 tumours (46.15%) were 1p35.1-p35.3. Two (15.38%) tumours with DNA copy number alterations were deleted at 1p12

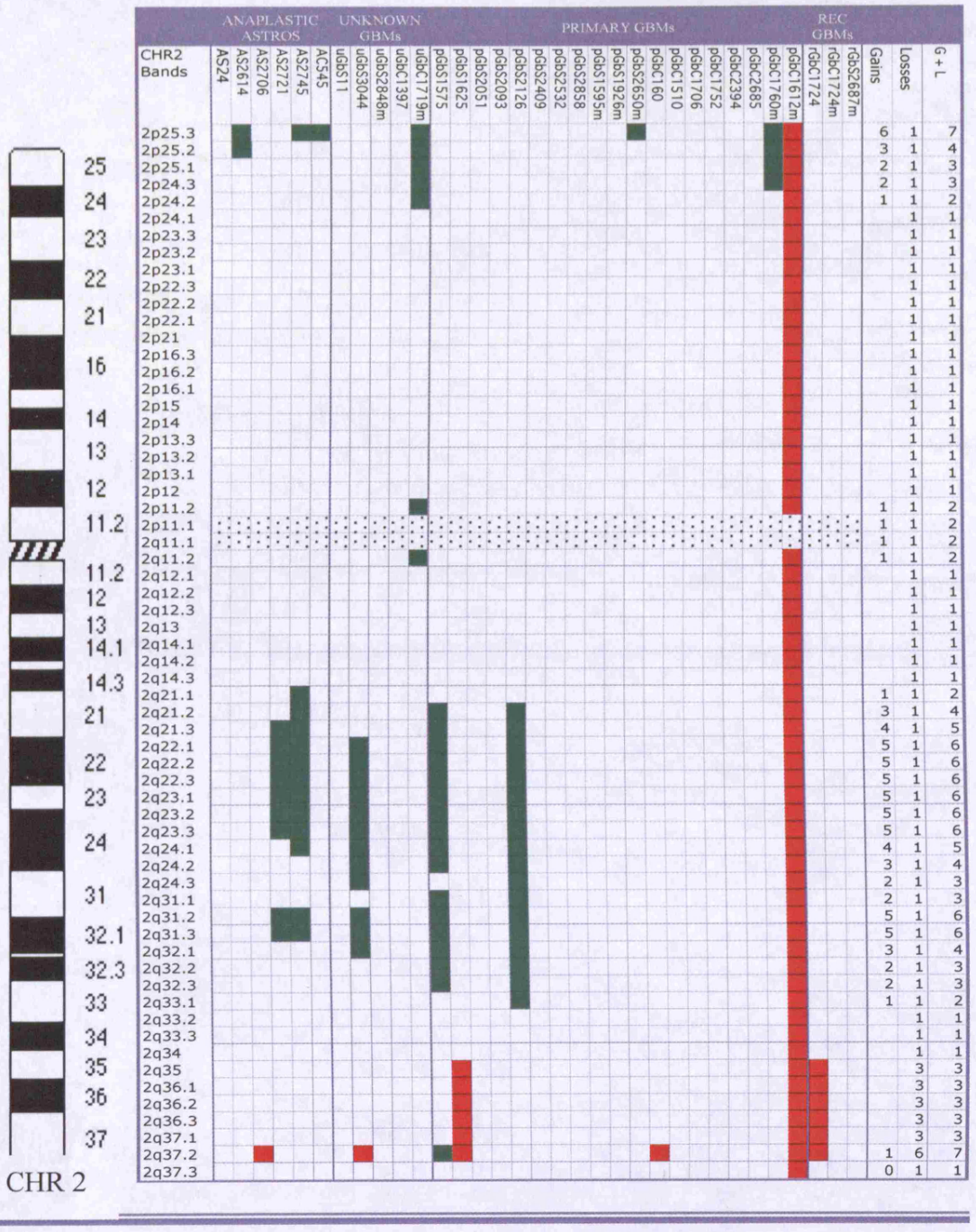
Deletions in the 1q-arm: Five (38.46%) tumours with DNA copy number alterations were deleted in the noncentromere heterochromatin band 1q12, but one of them, PGBS1926m, was deleted at 1p12-q22.

Genes of interest (GOIs) located in the most frequently deleted regions include the ones for cell division cycle 42 (CDC42 (1p36.1)), ephrin receptor A2 (EPHB2 (1p36.1-p35)), and paired box 7 (PAX7 (1p36.2-p36.12)), while the 4.9 Kb locus of nascient helix-loop-helix (NHLH) is at 1p12-p11.

Gains in the 1p-arm: Gains on chromosome 1 occurred in 10 (76.92%) informative cases. Several bands deleted in the p-arm, notably 1p32.3, 1p34.1 and 1p36.32-p36.3, are gained in a different set of tumours, RGBC1724 and PGBC1760m respectively. Gains in 8 out of 10 tumours (80%) were clustered at 1p13.2-1p31.2. The most commonly gained region is 1p22, which was gained in 7 (53.84) cases, i.e., PGBS1625 (1p22.2-p31.1), PGBS2051 (1p22.3), PGBS2126 (1p13.2-p31.2), UGBS3044 (1p13.2-p31.2), UGBC1397 (1p22.3) and RGBC1724 (1p13.2-p31.3).

The gene for the transcription factor wingless-type MMTV integration site, member 2B (WNT2B) is mapped to a 54.47 Kb locus at 1p13, while genes for bone morphogenetic protein, member 8B (BMP8B), paired box 7 (PAX 7), E2F transcription factor 2 (E2F2), natriuretic peptide precursor B (NPPB), and mitogen-activated protein kinase kinase kinase 6 (MAP3K6) are respectively mapped to loci at 1p35-p32, 1p36.2-p36.12, 1p36, 1p36.2 and 1p36.11.

Gains in the 1q-arm: Four tumours gained genetic material in the q-arm with two of them, UGBS3044 (1q24-q31) and PGBS2126 (1q31) sharing the gain at 1q31. The third and fourth tumour each had one isolated gain (7.69%), at 1q22-q23 for RGBC1724m, and at 1q43-q44 for PGBS1926m. The 305.09 Kb locus of astrotactin (ASTN1 (1q25.2) gene is mapped to 1q25.2, while the 89.68 Kb locus of the entactin or nidogen (NID1) gene is at 1q43.



3-4-2 Figure 3-10 - CHR 2: Ideogram and Excel chart of CNAs for 15 HGAs.

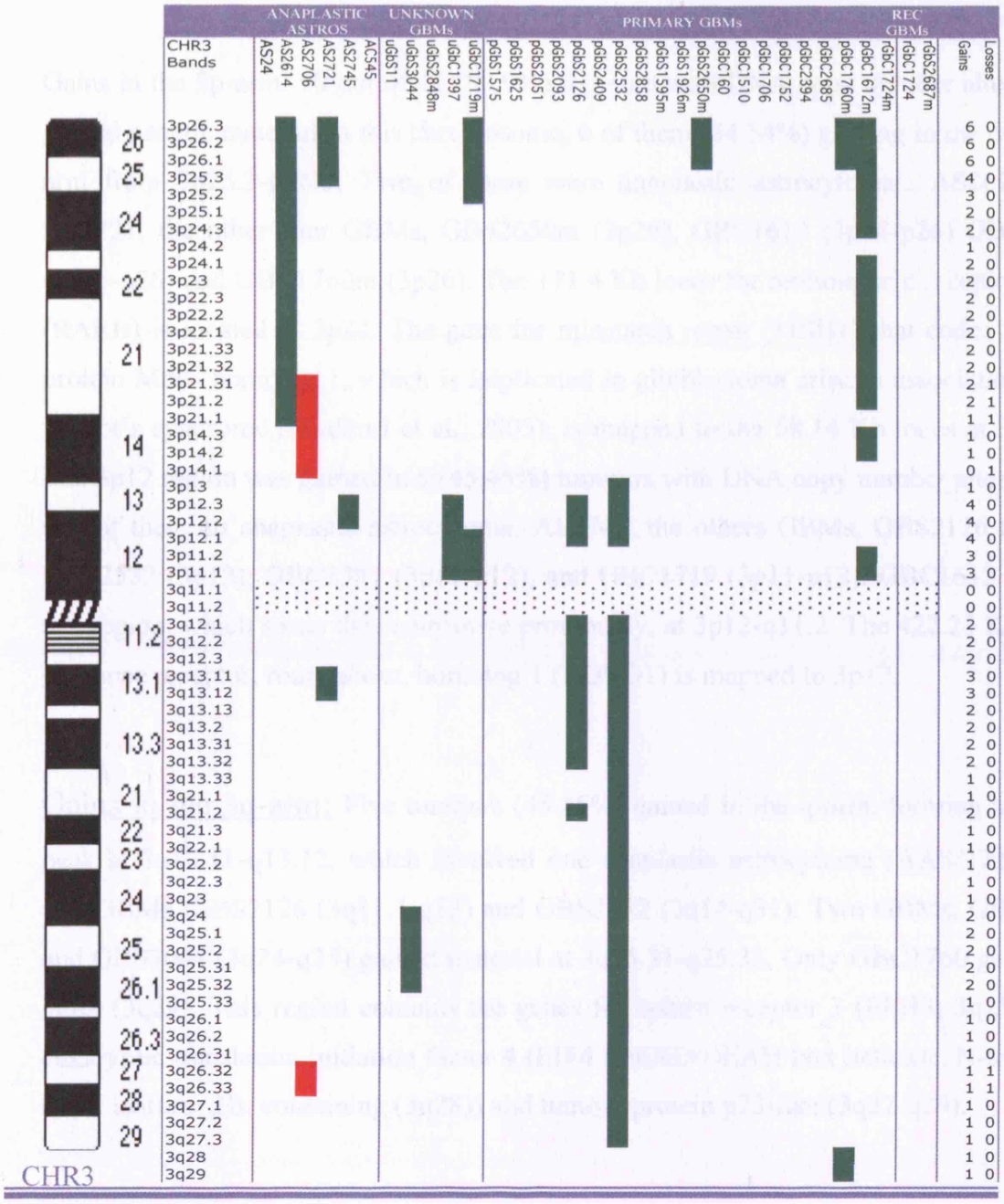
Fifteen tumours are informative for CNAs on this chromosome. One of them, PGBS1612m lost the entire chromosome 2, a finding that was confirmed in a repeat CGH experiment. This biopsy was taken at the initial operation, which was followed by recurrence and re-operation, when a second biopsy identified as RGBC1724 (RGBC1724m) was taken.

Deletions in the 2p-arm: Only one tumour, PGBC1612, lost the p-arm as part of an entire deletion of chromosome 2. The DNA mismatch repair gene, MutS homolog 2 (MSH2), which is believed to be involved in early stages of glioblastoma development (Wei et al., 1997), and in other solid tumours (Wei et al., 1997; Falkenback et al., 2005), is mapped to the 159.34 Kb locus at 2p22-p21.

Deletion in the 2q-arm: Six (40%) tumours with DNA copy number alterations lost genetic material; all but PGBC1612, which lost the entire chromosome (2p25-q37), were deleted only at the distal q-arm between 2q35-q37.3 - PGBS1625 (2q35-q37), RGBC1724 (2q35-q37), UGBS3044 (2q37), PGBC160 (2q37) and AS2706 (2q37) - making 2q37 the most frequently lost region in chromosome 2. The genes for insulin-like growth factor binding protein 5 (IGFBP5 (2q33-q36; 23.46 Kb)) and the paired box gene 3 (2q35-q37; 99.1Kb)) are located in the deleted region.

Gains in the 2p-arm: Six tumours (40%) gained material at the distal p-arm between 2p24.3 and 2p25.3, three were WHO malignancy grade 3 tumours, AS2614 (2p25), AS2745 (2p21-p24) and AC545 (2p25), the other three GBMs, UGBC1719m (2p24-p25), GBS2650 (2p25) and GBC1760m (2p24-p25); making 2p25 - pter the most commonly gained region in chromosome 2. The DEAD (Asp-Glu-Ala-Asp) box polypeptide 1 (DDX1 (2p24; 39.93 Kb)) and the N-myc and STAT interactor (NMI (2p24-q21.3)) genes are mapped within the amplified region. One tumour, UGBC1719m, gained genetic material across the centromere, at 2p11.2-2q11.

Gains in the 2q-arm: Five tumours with DNA copy number alterations (33.3%) gained genetic material over large segments in the middle of the q-arm between 2q21.1 and 2q33.3, creating peaks in 2 regions - 2q22-q23 (33.3%) and 2q31.2-q31.3 (33.3%). Two of these tumours were WHO malignancy grade 3, AS2721 and AS2745, and the other 3 were GBMs, PGBS1575, GBS2126, and UGBS3044. PGBS1575 also had a gain around 2qter (2q37). The genes for v-erb-a erythroblastic viral oncogene, homolog 4 (ERBB4; 2q33.3), postmeiotic segregation increased 1 (PMS1; 2q31-q33), neurogenin differentiation 1 (NEUROD1; 2q32) and paired box 3 (PAX3; 2q27) are mapped within the deleted region.

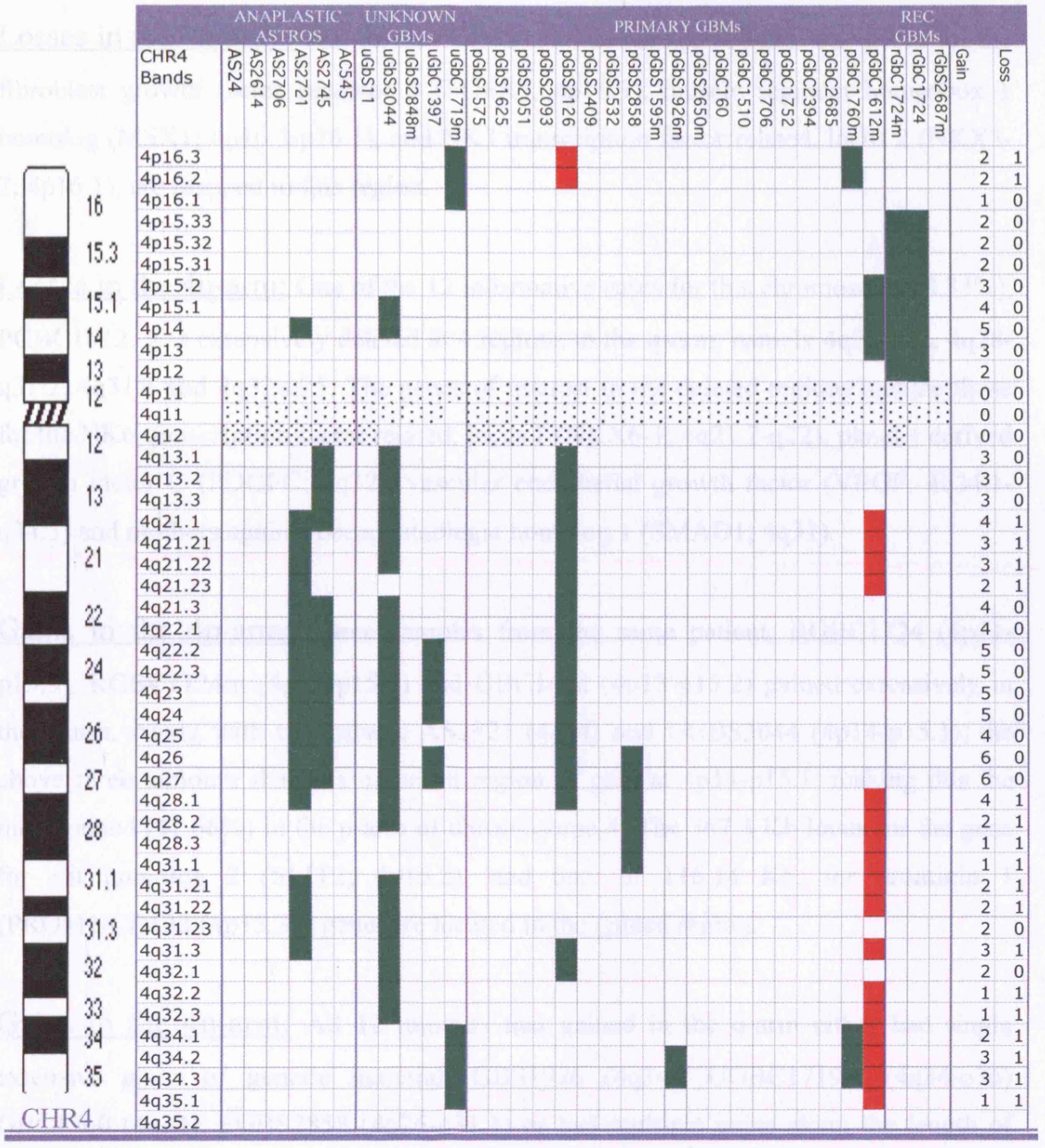


3-4-9 Figure 3-11 – CHR 3 Ideogram and Excel graph of CNAs for 12 HGAs

Losses at 3p- and q-arms: Only one of 12 (8%) tumours (AS2706) that were informative for this chromosome was deleted in both the p- (3p14-p21) and q-arms (3q26.3). The deletion in the p-arm spans the locus for the fragile site 3B (FRA3B), which contains the 333.85 Kb locus for the fragile histidine triad (FHIT.1) gene, which is mapped to 3p14.2, while, respectively, the 31.23 Kb and 60.66 Kb loci for the genes for cell division cycle 25A (CDC25A) and (CTNNB1) are mapped to 3p21.

Gains in the 3p-arm: 10 out of 11 (90.9%) tumours with DNA copy number alterations gained genetic material in this chromosome, 6 of them (54.54%) gaining in the distal p-arm from 3p25.3-p26.3. Two of these were anaplastic astrocytomas, AS2614 and AS2721, the other four GBMs, GBS2650m (3p26), GBC1612 (3p24-p26) GBC1719 (3p25-p26) and GBC1760m (3p26). The 171.4 Kb locus for retinoic acid receptor, beta (RARB) is located at 3p24. The gene for mismatch repair (MSH1) that codes for the protein MutL homolog 1, which is implicated in glioblastoma arise in association with Turcot's syndrome (Madhuri et al., 2005), is mapped to the 58.14 Kb locus at 3p21.3. The 3p12 region was gained in 5 (45.45%) tumours with DNA copy number alterations, one of them an anaplastic astrocytoma, AS2745, the others GBMs, GBS2126 (3p12), GBS2532 (3p12), GBC1397 (3p11-p12), and GBC1719 (3p11-p12). GBC1612 gained at a region, which spans the centromere proximally, at 3p12-q11.2. The 422.23 Kb axon guidance receptor, roundabout, homolog 1 (ROBO1) is mapped to 3p12.

Gains in the 3q-arm: Five tumours (45.45%) gained in the q-arm, forming a minor peak at 3q13.11-q13.12, which involved one anaplastic astrocytoma (AAS/2721) and two GBMs, GBS2126 (3q11.1-q13) and GBS2532 (3q14-q31). Two GBMs, GBS2532 and GBS3044 (3q24-q25) gained material at 3q25.31-q25.33. Only GBC1760 gained at 3qter (3q29). This region contains the genes for ephrin receptor 3 (EPH3; 3q21-qter), eukaryotic translation initiation factor 4 (EIF4 (DEAD/DEAH box helicase, N-terminal motif isoform 2B, containing (3q28)) and tumour protein p73-like (3q27-q29).



3-4-10 Figure 3-12 – CHR 4: Ideogram and Excel graph of CNAs for 12 HGAs.

Chromosome 4 showed more gains than losses, with 7 out of (12 ~58%) informative cases gaining and only one (8.33%), PGBS2126 was deleted in the p-arm. Large gains occurred in the q-arm in 9 out of 12 (75%) informative cases; only one tumour (8.33%) was deleted extensive in the q-arm.

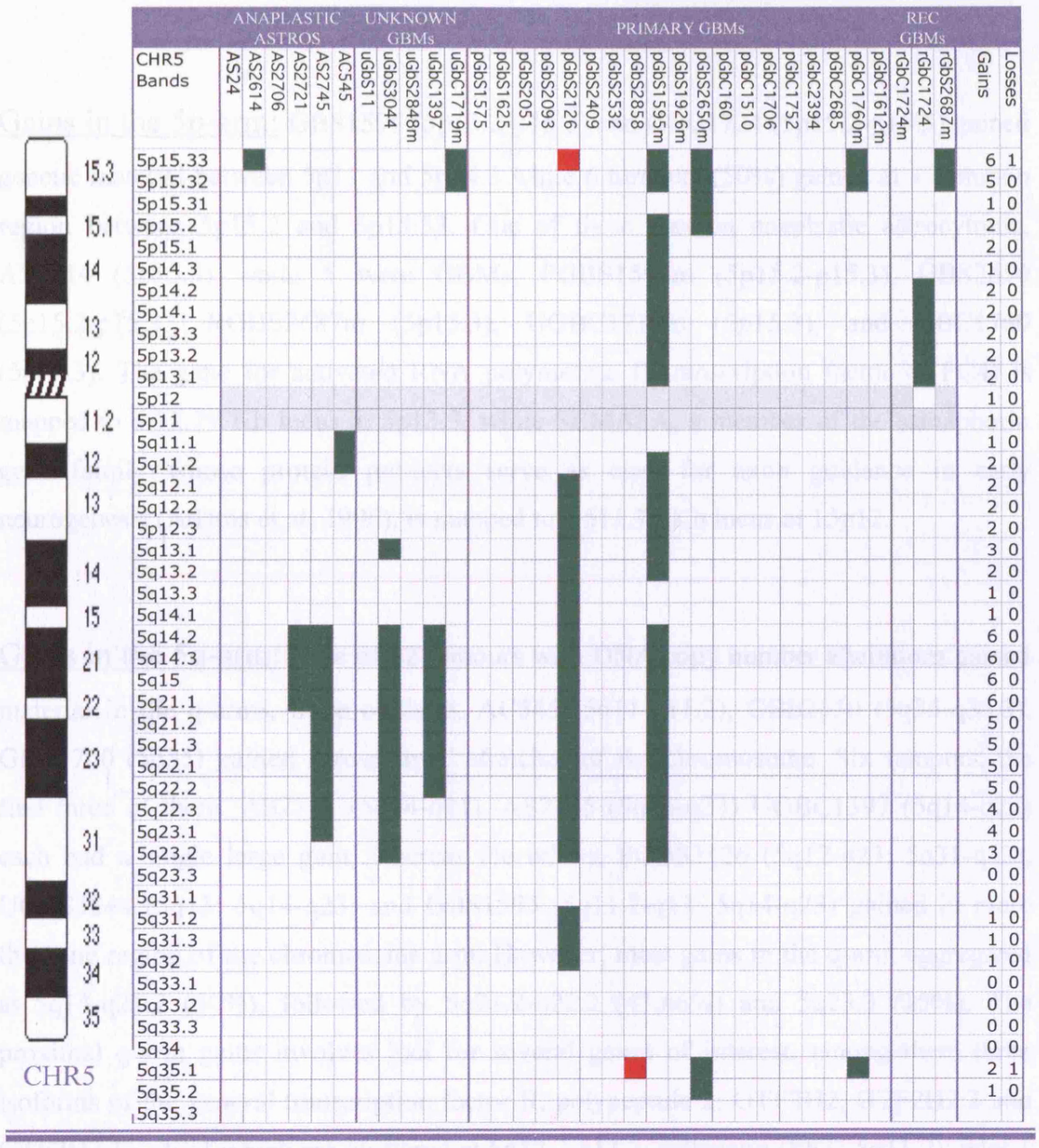
Losses in the 4p-arm: One GBM, PGBS2126 was deleted at 4p16. The genes for the fibroblast growth factor receptor 3 (FGFR3, 4p16.3), muscle segment homeobox 1 homolog (MSX1; 4p16.3-p16.1), and NK3 transcription factor related, locus 2 (NKX3-2; 4p16.1), are mapped to this region.

Losses in the 4q-arm: One of the 12 informative cases for this chromosome (8.33%), PGBC1612, was extensively deleted at 4 regions in the q-arm, namely 4q21-q22, 4q28-q31.2, 4q31.3 and 4q32-q35. The genes of interest in the deleted regions include those for the NK6 transcription factor related, locus 1 (NKX6-1; 4q21.2-q22), platelet derived growth factor C (PDGFC; 4q32), vascular endothelial growth factor (VEGF; 4q34.1-q34.3) and mothers against decapentaplegic homolog 1 (SMAD1; 4q31).

Gains in the 4p-arm: Three samples from the same patient, RGBC1724 (4p11-p15.3), RGBC1724m (4p12-p15.3) and GBC1612 (4p13-p15.2) gained extensively in the p-arm. Along with two others, AS2721 (4p14) and UGBS3044 (4p14-p15.1), the above three tumours shared a common region of gain at 4p14-p15.1, making this the most gained (41.66%) in the p-arm of chromosome 4. The 367.3 Kb locus for the gene for slit homolog 2 (SLIT2; 4p15.2), and one, of 116.14 Kb, for prominin 1 (PROM1/CD133 (4p15.32) gene, are located in the gained region.

Gains in the 4q-arm: All 12 tumours that gained in the q-arm either had single extensive gains of genetic material, GBS1926 (4q34), UGBC1719m (4q34-q36) GBC1760 (4q34), PGBS2858 (4q26-q31.1) or had multiple gains along the length of the chromosome arm: UGBC1397 (4q22-q24; 4q26-q27), PGBS2126 (4q13-q28; 4q31.3-q32), and UGBS3044 (4q12-q21; 4q21-q28; 4q31.2-q32). Two out of 12 tumours that are informative for this chromosome arm (16.66%), AS2721 (4q21-q28; 4q31.2-q31.3) and AS2745 (4q13-q21; 4q22-q27) are anaplastic astrocytomas, while nine (75%) are GBMs. The gains span respectively the 83.25 and 47.45 Kb loci of genes for CKIT, the human homolog of the proto-oncogene c-kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) and vascular endothelial growth factor receptor or kinase insert domain receptor (VEGFR/KDR), which are both mapped to 4q11-q12.

The two most frequently gained regions in the q-arm were 4q22-q24 (41.66%) and 4q26-q27 (58.33%). These regions span the loci for endomucin (EMCN, 4q24) and epidermal growth factor (EGF; epidermal growth factor (beta-urogastrone); 4q25)) genes.



3-4-11 Figure 3-13 – CHR 5: Ideogram and Excel graph of CNAs for 14 HGAs.

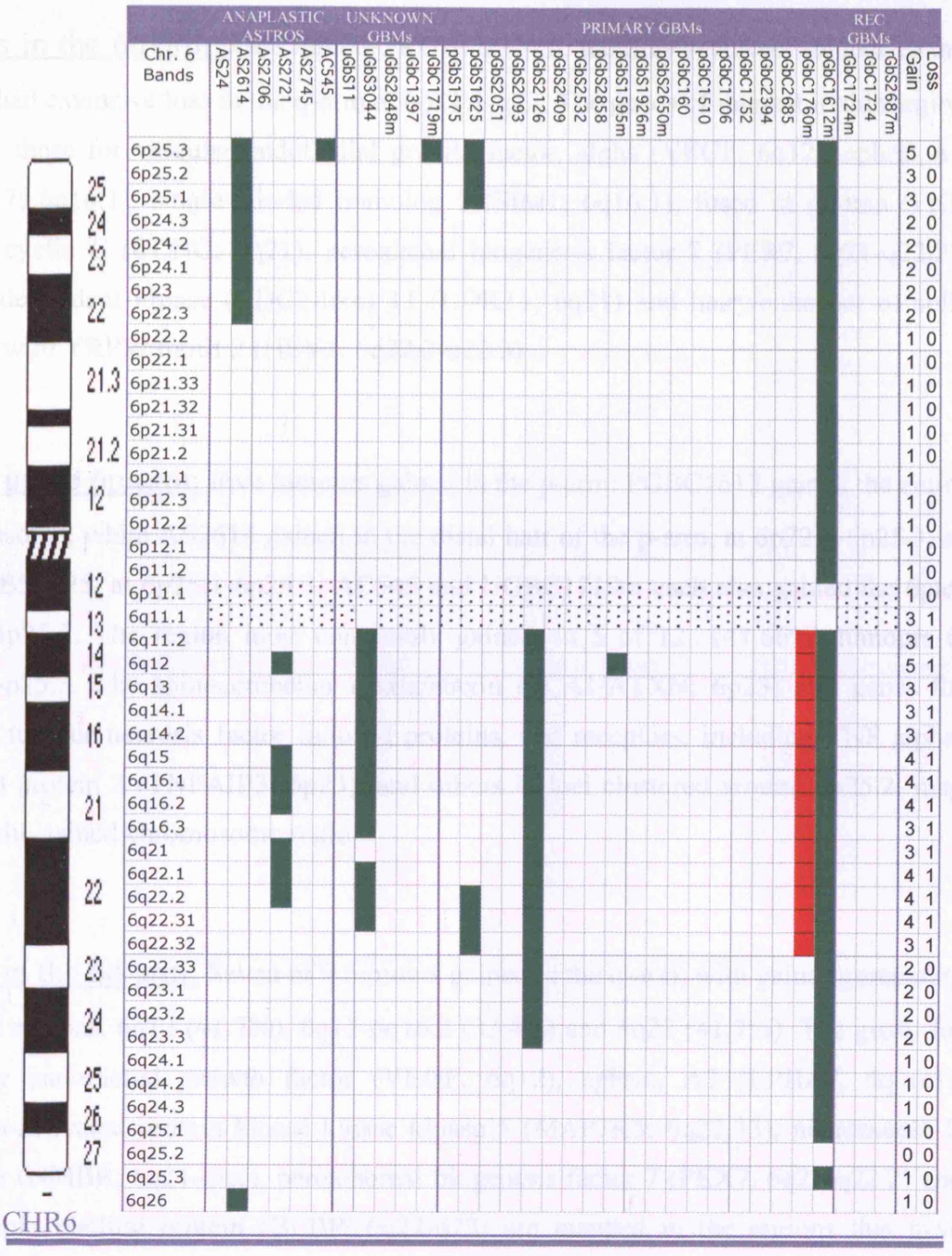
Chromosome 5 had more gains than losses among 14 informative cases for this chromosome. There was one prominent region of gain at the distal p-arm and one located midway along the q-arm

<u>Losses in the 5p:</u> Two GBMs were deleted, one, PGBS2126, at 5p15.33 and PGBS2858 at 5q35.2. The muscle segment homeobox homolog 2 (MSH2, 5q34-q35) and the slit 3 (SLIT3; 5q35) genes are located within the q-arm deleted region.

Gains in the 5p-arm: GBS1595 (5p15.2-p15.3) and GBC1724 (5p15.2-p15.3) gained genetic material between 5p11 and 5p14.3 while 6 tumours (50%) gained at a common region between 5p15.2 and 5p15.33. One of these was an anaplastic astrocytoma, AS2614 (5p15.3) while 5 were GBMs, PGBS1595m (5p15.2-p15.3), GBS2650 (5p15.2-p15.3), RGBS2687m (5p15.3), UGBC1719m (5p15.3), and GBC1760 (5p15.3). The gene for activated RNA polymerase II transcription factor 4 (PC4) is mapped to a 72.29 Kb locus at 5p13.3, while SEMA5A, a member of the samaphorin gene family whose protein products serve as cues for axon guidance in early neurogenesis (Adams et al. 1996), is mapped to a 511.75 Kb locus at 15p12.

Gains in the 5q-arm: Nine of 12 tumours with DNA copy number alterations gained material in the q-arms, three of them, AC545 (5q11-q11.2), GBS2650 (5q35-q35.3), GBC1760 (5q35) gained across small stretches of the chromosome. Six tumours, the first three of them, AS2721 (5q14-q21), AS2745 (5q14-q23) UGBC1397 (5q14-q22) each had a single large gain, whereas the others, PGBS2126 (5q12-q23; 5q31-q32), UGBS3044 (5q13; 5q14-q23) and GBS1595 (5q11.2-q13; 5q14-q23) gained in more than one region of the chromosome arm. However, most gains in the q-arm aggregated at 5q14-q21.2 (50%), followed by 5q21.2-q22.2 (41.66%) and 5q23.3 (25%). The proximal q-arm gains involves loci for several genes of interest, among them three isoforms of the general transcription factor II, polypeptide 2: GTF2H2, GTF2H2.2 and GTF2H2.1 - all of which are clustered at 5q12.2-q13.3; follistatin (FST; 5q11.2); islet-1 transcription factor, LIM/homeo domain (ISL1; 5q11.2) and cyclin H (CCNH; 5q13.3-q14). Five tumours have gains, which span the 185.81 Kb locus at 5q22, for the adenomatosis polyposis coli gene (APC), which is implicated in colon cancer (Sarova et al., 1998). The locus for the cell division cycle 25C (CDC25C) is mapped to distal q-arm at 5q31, and is gained in PGBS2126.

Other genes of interest located in the 5q-arm are, the genes for ephrin-A5 (EFNA5; 5q21), neurogenin 1 (NEUROG1; 5q23-q31), fibroblast growth factor (FGF; 5q31), and platelet derived growth factor receptor (PDGFR; 5q31-q32).



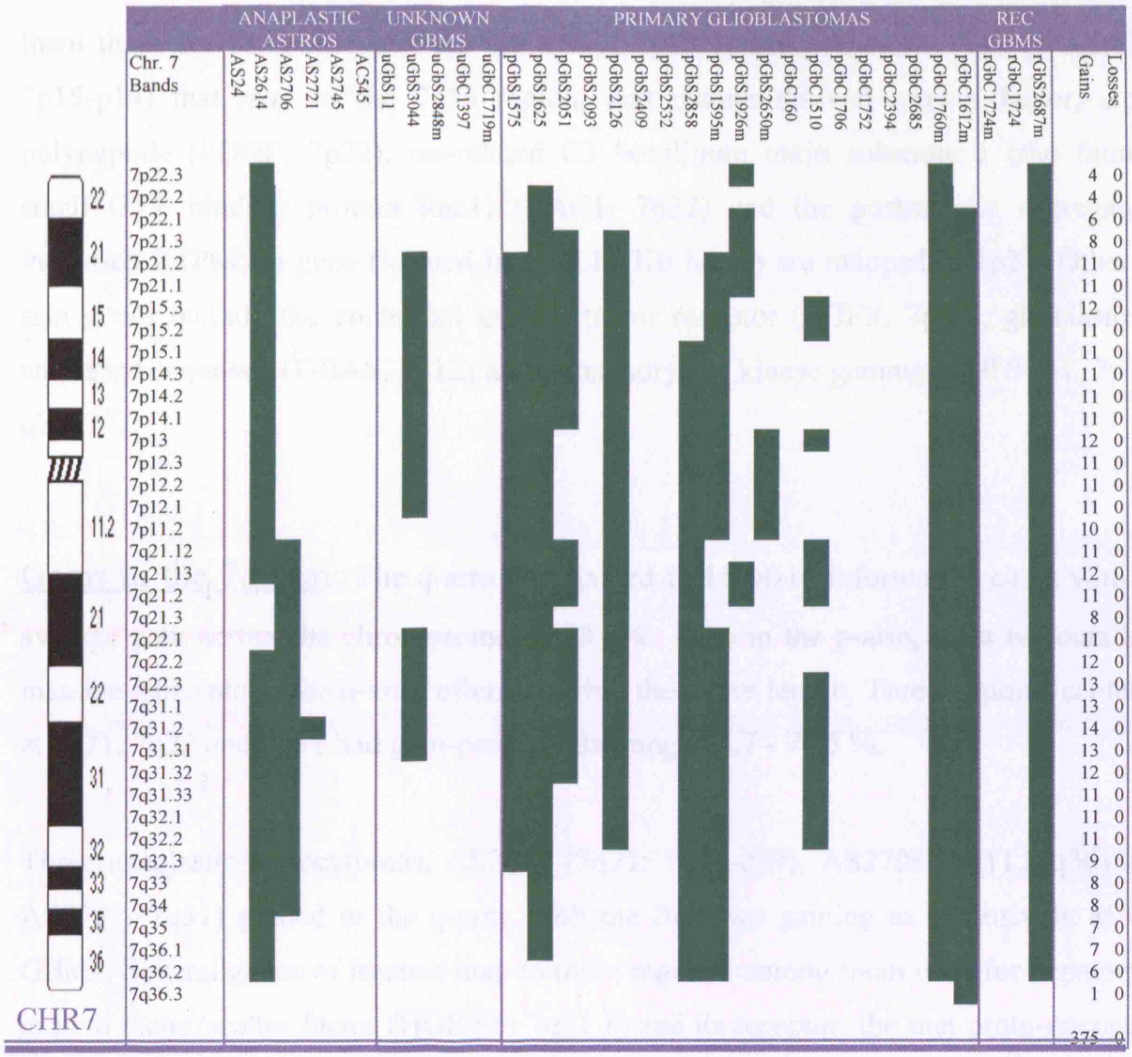
3-4-12 Figure 3-14 – CHR 6: Ideogram & Excel graph of CNAs for 9 HGAs.

Nine tumours (31.25%) showed DNA copy number changes on this chromosome with all except PGBC1612 gaining genetic material, 5 of them in the p-arm, and 7 in the q-arm. PGBC1612 gained nearly the entire chromosome 6 (6p25-6q25 and 6q26).

Losses in the 6q-arm: One tumour, RGBC1724m, the only recurrent tumour in the study, had extensive loss in the q-arm at 6q11.1-q22. The genes of interest in this region include those for vascular endothelial growth factor, alpha (VEGF, 6q12), ephrin A7 (EPHA7; 6q16.1), single-minded homolog 1 (SIM1; 6q16.1), fused in glioma (FIG; 6q21), cyclin C (CCNC; 6q21), peroxismal biogenesis factor 7 (PER7, 6q21-q22.2), cyclin dependent kinase (CDC2-like) 11 (CDK11; 6q21) and hairy/enhancer of split related with YRPW motif 2 (HEY2; 6q22.2-q22.3).

Gains in the 6p-arm: Five tumours gained in the p-arm: PGBC1612 gained the entire chromosome, while AS2614 gained in the distal half of the p-arm, at 6p22.3-6p25.3, as did PGBS1625, at 6p25.1-6p25.3. AC545 and UGBC1719m each also gained the 6pter band, 6p25.3. The region most commonly gained, in 5 of 12 (41.66%) tumours is 6p25.1-p25.3. The spinocerebellar ataxia/ataxin (SCA1/ATXN; 6p23) and genes for several tumour necrosis factor induced proteins, and receptors, including TNF alpha-induced protein 3 (TNFAIP3; 6p23), and others in loci clustered around 6p25.2, map within the gained chromosome region.

Gains in the 6q-arm: Seven of 9 tumours gained in the q-arm, with gains aggregating in three regions, 6q12 (41.7%), 6q15-6q16.2 (33.4%) and 6q22 (41.7%). The genes for vascular endothelial growth factor (VEGF, 6q12), ephrin, A7 (EPHA7, 6q16.1), mitogen-activated protein kinase kinase kinase 5 (MAP3K5, 6q22.33), neuromedin B receptor (NMBR, 6q21-qter), peroxisomal biogenesis factor 7 (PEX7, 6q21-q22.2) and brain lipid binding protein (BLBP, 6q22-q23) are mapped in the regions that have gained. Three tumours gained genetic material in the q-arm region, which spans the locus for the oligodendrocyte transcription factor 3 (OLIG3) at 6q23.3, which is also the location of the transcription factor 21 gene (TCF21) that codes a protein with DNA dependent transcription- and RNA II transcription factor activity (Hidai et al., 1998) and has a role in lineage specific differentiation early in embryonic development (Hidai et al., 1998; Funato et al. 2003).



3-4-13 Figure 3-15 – CHR 7: Ideogram and Excel graph of CNAs for 16 HGAs.

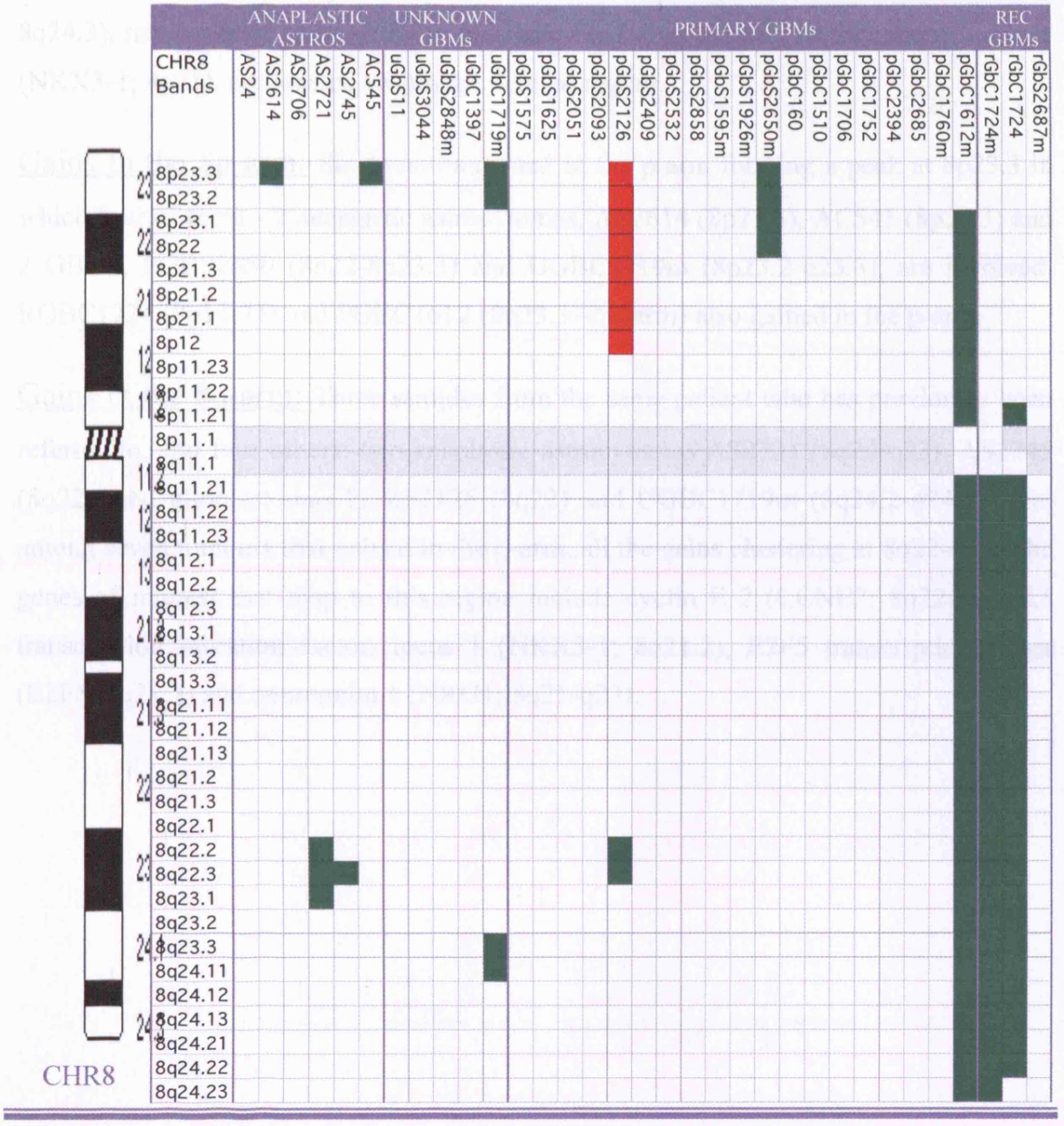
16 tumours (51.5%) are informative for CNAs on this chromosome; all are gains in one or both chromosome arms. Except for AS2721, all informative samples showed extensive gains covering large portions of the chromosome arms. In most cases, the entire length of an arm and the entire chromosome gained genetic material. Remarkably, this chromosome did not show any loss of genetic material.

<u>Gains in the 7p-arm:</u> 11 out of 16 tumours (68.8%) gained material along the entire p-arm except at p-ter bands (7p22.1-p22.3) where the average gain was 29.4%. Two regions, 7p13-p14.1 and 7p15.1-p15.3, were gained in 70.5% of the tumours. One of the five anaplastic astrocytomas in the study (20%), AS2614, gained genetic material in the entire p-arm. A number of genes of interest are located in the gained regions, among

them those for the homeobox A1 (HOXA1; 7p15.3) and homeobox A13 (HOXA13; 7p15-p14) that map to the 7p15 region, and platelet-derived growth factor, alpha polypeptide (PDGF, 7p22), ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) (RAC1, 7p22) and the postmeiotic segregation increased 2 (PMS2) gene (located in a 38.18 Kb locus) are mapped at 7p21. Other p-arm genes include the epidermal growth factor receptor (EGFR, 7p12), glioblastoma amplified sequence (GBAS, 7p12) and phosphorylase kinase gamma 1 (PHKG1, 7p12-q21).

Gains in the 7q-arm: The q-arm was gained in 15 of 16 informative cases with an average gain across the chromosome of 58.8%. Like in the p-arm, most tumours had massive gains along the q-arm, often covering the entire length. Three regions, centered at 7q21, 7q22 and 7q31 had gain-peaks in the range 64.7 - 70.5 %.

Three anaplastic astrocytomas, AS2614 (7q21; 7q22-q27), AS2706 (7q11.2-q36) and AS2721 (7q31) gained in the q-arm, with the first two gaining as extensively as did GBMs. Several genes of interest map to these regions, among them ones for hepatocyte growth factor/scatter factor (HGF/SF; 7q21.1) and its receptor, the met proto-oncogene (hepatocyte growth factor receptor (HGFR/MET/CMET; 7q31)), phosphoinisitide-3-kinase, catalytic gamma polypeptide (PI3K; 7q22.3), fragile site 7G (FRA7G; 7q31.2) and Wiscott-Aldrich syndrome-like (N-WASP/WASL; 7q31.3). The CHR region 7q32.3 is gained in 12 out of 17 tumours with DNA copy number alterations. This region is known to harbour locus for SMO (Smothered), a receptor for Patched (PTH), which is implicated in the oncogenesis of ependymomas.



3-4-14 Figure 3-16 – CHR 8: Ideogram and Excel graph of CNAs for 10 HGAs.

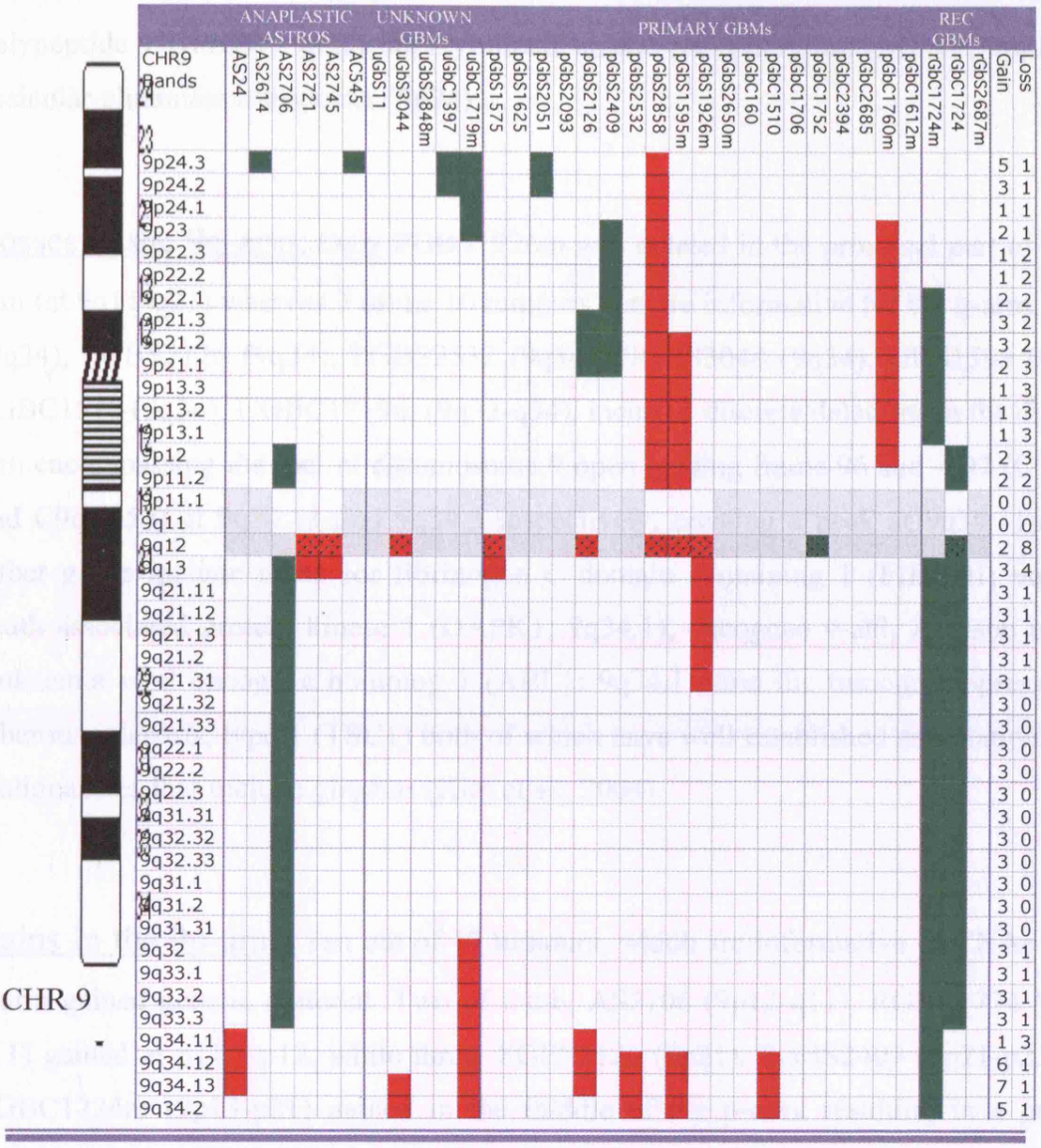
Nine tumour samples were informative for this chromosome. Except for three tumours from the same patient, the other five samples had small gains in a corresponding region at the distal portion of the p-arm in 4 out of 5 cases, and in the region 8q22 in 3 of 5 cases. Only one sample out of nine had a deletion, which was in the p-arm.

Losses in the 8p-arm: PGBS2126 lost a large portion of the p-arm at 8p12-p23. The genes for scleraxis, the basic helix loop helix dimerisation region 2 (SCX/bHLH.2;

8q24.3), neuregulin 1 (NRG1; 8p21-p22) and NK3 transcription factor related, locus 1 (NKX3-1; 8p21), are located within the deleted region.

Gains in the 8p-arm: Six tumours gained in the p-arm forming a peak at 8p23.3 in which four of them - 2 anaplastic astrocytomas, AS2614 (8p23.3), AC545 (8p23.3) and 2 GBMs, PGBS2650 (8p22-8p23.3) and UGBC1719m (8p23.2-p23.3), are involved. RGBC1724 (8p11.21) and PGBC1612 (8p23.3 –>q-arm) also gained in the p-arm.

Gains in the 8q-arm: Three samples from the same patient who has previously been referred to, and four others: two anaplastic astrocytomas AS2721 (8q22-q23), AS2745 (8q22), and glioblastomas PGBS2126 (8q22) and UGBC1719m (8q24.2-q24.3) were among seven tumours that gained in the q-arm, all the gains clustering at 8q22-q23. The genes of interest that map to this region include cyclin E 2 (CCNE2; 8q22.1), NK3 transcription initiation factor, locus 1 (NKX3-1; 8q21.2), E2F5 transcription factor (E2F5; 8q21.2) and neuregulin 1 (NRG1; 8q21-q22).



3-4-15 Figure 3-17 – CHR 9: Ideogram and Excel graph of CNAs for 22 HGAs.

Twenty-two tumours showed DNA copy number changes in this chromosome, a number of them have losses or gains in either arm.

Losses in the 9p-arm: Three GBMs, PGBS2858 (9p24-q12), GBS1595 (9p12-p21) and GBC1760 (9p12-p23) had deletions spanning the centromere and adjacent heterochromatin band, which extend to involve large parts of the p-arm. Genes of interest include those for cyclin dependent kinase 2A (p16) / cyclin dependent kinase 2B/(p14ARF) / p15 / inhibitor of CDK4 (CDKN2A/CDKN2B); 9p21); interferon, beta

polypeptide 1 (IFNB1, 9p21), methylthioadenosine phosphorylase (MTAP; 9p21) and vesicular glutamate transporter (9p24).

Losses in the 9q-arm: Only PGBS1926m was deleted in the proximal part of the q-arm (at 9q11-q21) whereas 7 of the 10 tumours that are informative for the q-arm, AS24 (9q34), PGBS2126 (9q34), PGBS2532 (9q34), UGBS3044 (9q34), GBS1595 (9q34), PGBC1510 (9q34), UGBC1719m (9q33-q34), incurred discrete deletions in the distal q-arm encompassing the loci of chromosome 9 open reading frame 96 and -197 (C9orf96 and C9orf157) at 9q34.11 and 9q34.3 respectively, creating a peak at 9q34.11-q34.2. Other genes include those for fibrinogen C domain containing 1 (FIBCD1; 9q34.2), death associated protein kinase 1 (DAPK1; 9q34.1), oncogene v-abl, Abelson murine leukaemia viral oncogene homolog 1 (ABL1; 9q34.1), and the tumour suppressor for tuberous sclerosis, type 1 (TSC1) both of which have well established associations with malignancies that include gliomas (Kim et al., 2004).

Gains in the 9p-arm: Ten out of 17 tumours, which are informative of CNAs in the p-arm gained genetic material. Two of these, AS2706 (9p12-q11), RGBC1724 (9p13-q11) gained at p11.1-p12, while three, PGBS2126 (9p21), PGBS2409 (9p21-p23) and RGBC1724m (9p13-p21) gained in the middle of the p-arm, resulting in a peak at 9p21.1-9p21.3. However, 5 tumours, AS2614 (9p24), AC545 (9p24), PGBS2051 (9p24), UGBC1397 (9p24) and UGBC1719M (9p23-p24) gained in the distal p-arm-bands, forming a prominent peak at 9p24.2-p24.3. The genes for CDKN2A/CDKN2B, interferon beta 1 (IFNB1), methylthioadenosine phosphorylase (MTAP) and DBRT-like family 1 (DMRTA1; 9p21.1) are located at 9p21, whereas the gene for solute carrier family 1, member 1 (SLC1A1), the neuronal/epithelial high affinity glutamate transporter, maps to the 97.26 Kb locus at 9p24.

Gains in the 9q-arm: Two of the three tumours that gained in the q-arm, AS2706 (9q21-q34), RGBC1724 (9p12-q33), RGBC1724m (9q13-q34) are from the same patient. All but RGBC1724m gained the entire q-arm from the centromere downwards, truncating at 9q33.3. The gained region contains the genes for the human homolog of

patched (PTCH; 9q22), which is a receptor for sonic hedgehog, astrotactin 2 (ASTN2; 9p33.1) and fibrinogen C domain containing 1 (FIBCD1; 9p34.2).

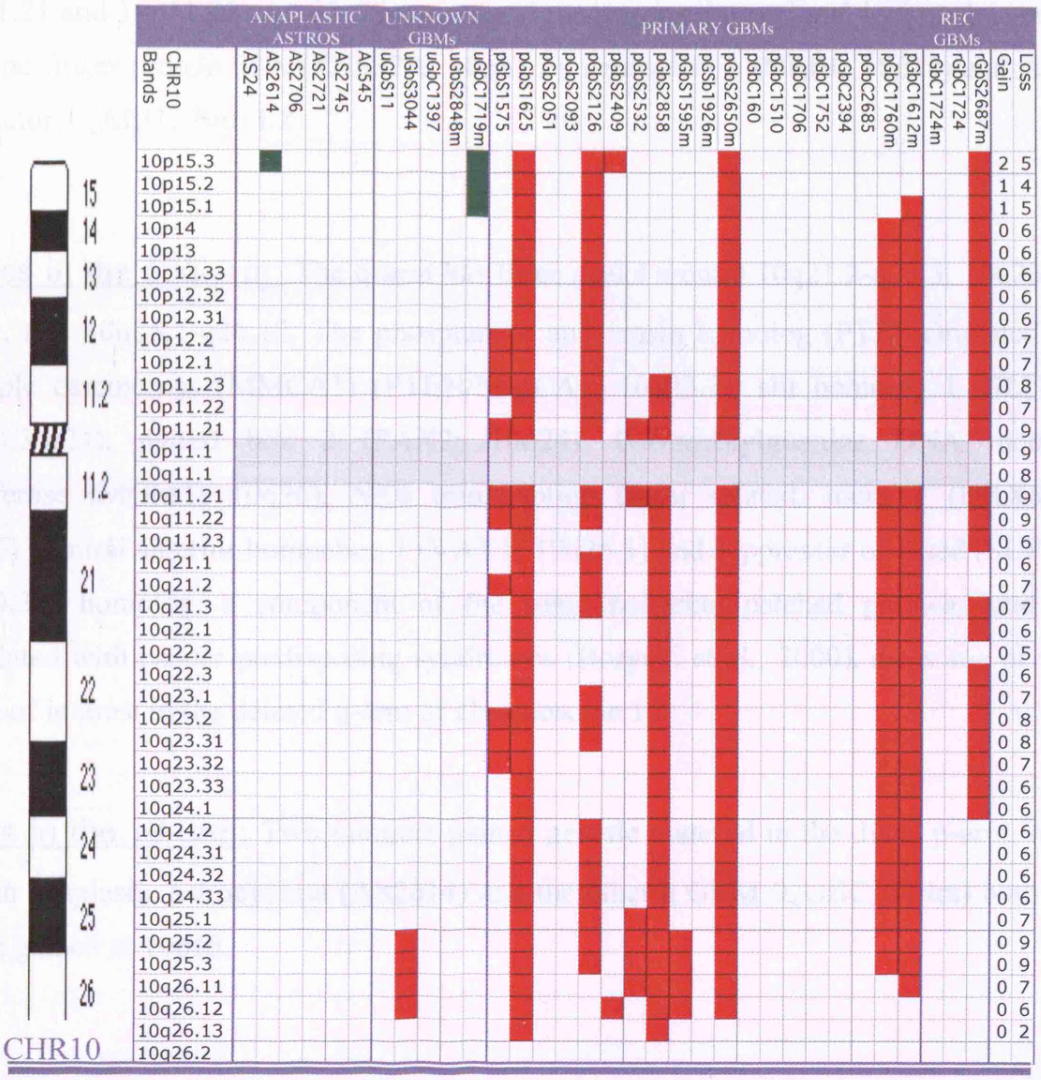


Figure 3-18– CHR 10: Ideogram & Excel graph of CNAs for 14 HGAs

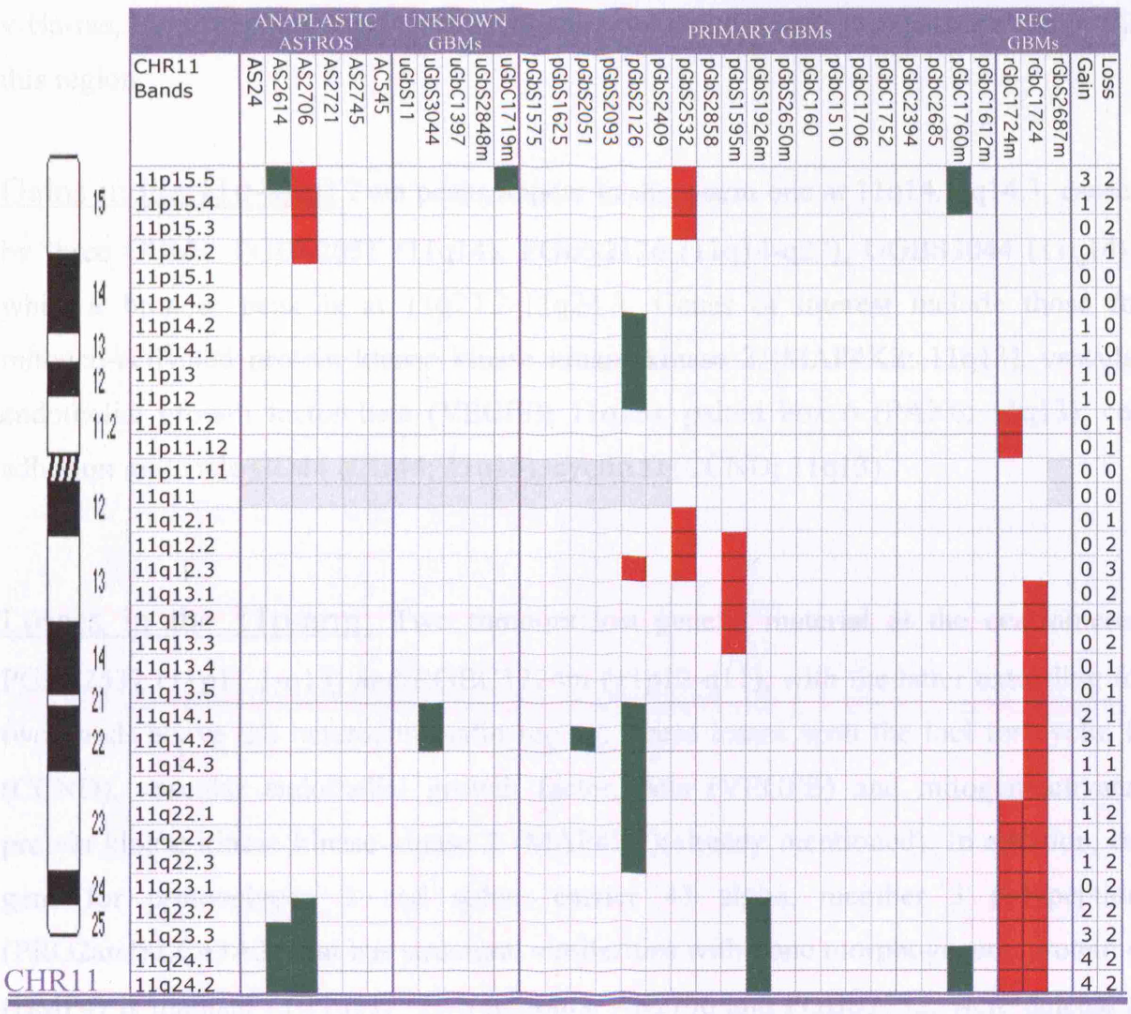
Fourteen tumours showed DNA copy number changes in this chromosome. Except for two, AS2614 (10p15) and UGBC1719m (10p15), which gained genetic material at the distal p-arm, and four, PGBS2409 (10p15; 10q26), PGBS2532 (10p11.2-q11.2; 10q25-q26) UGBS3044 (10q25-q26) GBS1595 (10q24-q25) that were deleted in relatively shorter stretches of the chromosome, the rest namely GBS1575, PGBS1625, PGBS2126, PGBS2858, GBS2560m, RGBS2687m, PGBC1612, PGBC1760m had multiple patchy losses over long sections, and whole chromosome arms or the entire

chromosome. The average loss across the length of chromosome 10 in the informative group is approximately 70%.

Losses in the 10p-arm: There are two deletion peaks in the p-arm, located at 10p11.21 and 10p11.22-p11.23. The proximal peak spans the regional loci of the genes for zinc finger protein 33A (ZNF33A; 10p11.2), neuropilin 1 (NRP1; 10p12) and Abl-interactor 1 (ABI1; 10p11.2).

Losses in the 10q-arm: The q-arm has three peaks around 10q21.2-q21.3, 10q23.1-q23.3, and 10q25.1-q26.12. The phosphatase and tensin homolog (PTEN)/mutated in multiple carcinomas (MMCA1) (PTEN/MMCA1; 10q23.3), slit homolog 1 (SLIT1; 10q23.3-q24), paired box 2 (PAX2; 10q24), O-6-methylguanine DNA methyl transferase (MGMT; 10q26), NK6 transcription factor related, locus 2 (NKX6.2; 10q26), ventral anterior homeobox 1 (VAX1; 10q26.1) and suppressor of fused (SUFU; 10q24.32) homolog, a component of the sonic hedgehog/patched pathway and is associated with cancer predisposing syndromes (Borycki et al., 2000), are some of the genes of interest in the deleted q-arm of chromosome 10.

Gains in the 10-arm: Two tumours gained genetic material in the distal p-arm; one was an anaplastic astrocytoma (AS2614) and the other a GBM (UGBC1719m) both of which gained at 10p15.



3-4-16 Figure 3-19 – CHR 11: Ideogram and Excel graph of CNAs for 13 HGAs

12 tumours are informative for CNAs in this chromosome, but only 5 of them were highlighted in the p-arm.

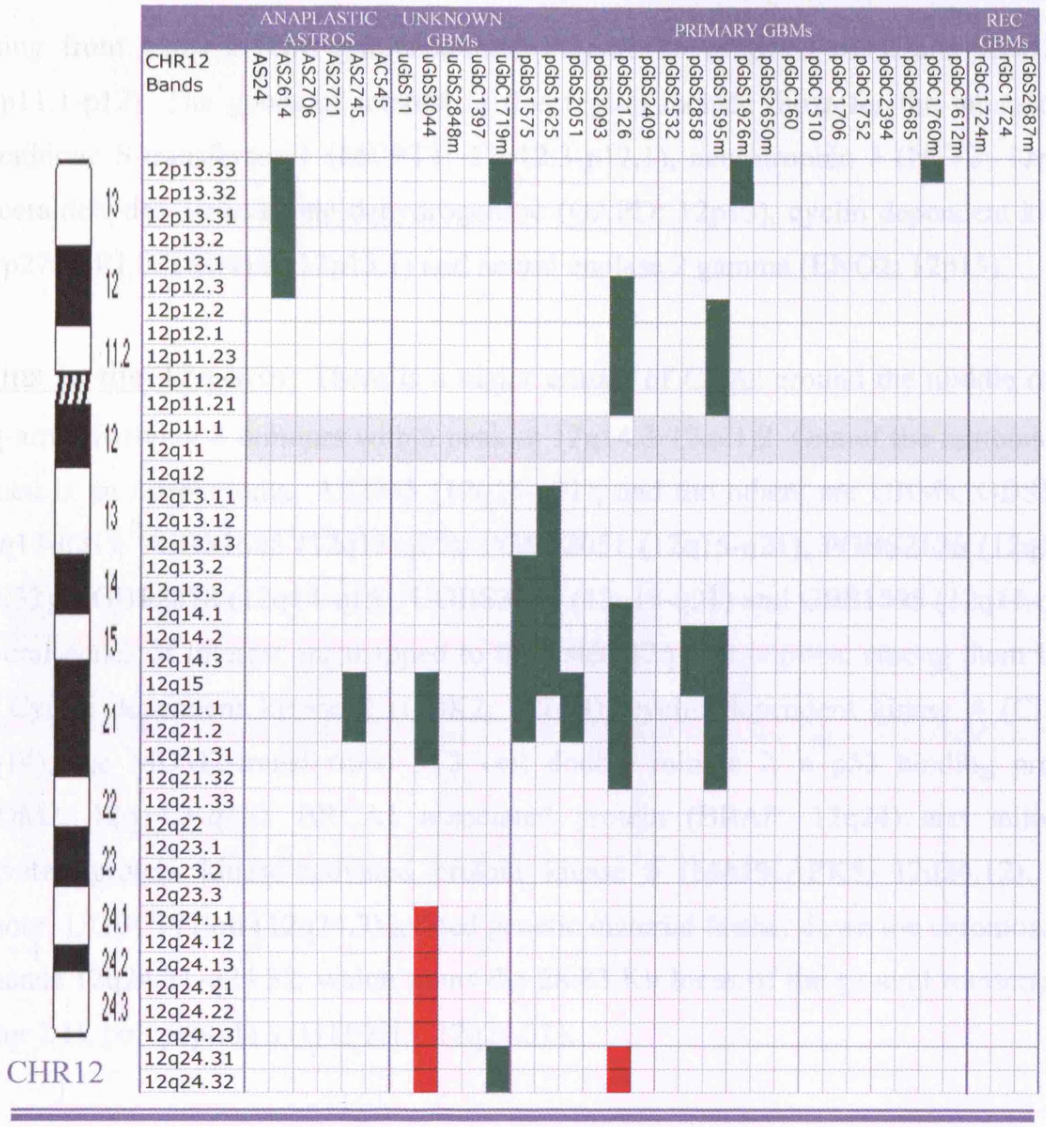
<u>Gains in the 11p-arm:</u> A minor peak of gain was formed at 11p15.5, arising from gains in one anaplastic astrocytoma, AS2614 (11p15) and two glioblastomas, UGBC1719m (11p15) and PGBC1760m (11p15), while another glioblastoma, PGBS2126, gained genetic material proximally, at 11p12-11p14.2. The genes for transcription factor 2H, member 1 (TFIIH1; 11p15.3), achaete-scute complex like 3 (ASCL3), myogenic factor 3 (MYOD1; 11p15.4), hypothetical protein LOC441583.1 (similar to radixin (ESP10; 11p15.4), that codes for a member of the ERM protein family, a E2F–related hypothetical protein, FLJ23311 (11p15.1) and the proto-oncogene

v-Ha-ras, Harvey rat sarcoma viral oncogene homolog (HRAS; 11p15.4) are mapped in this region.

Gains in the 11q-arm: Two peaks appear in the q-arm one at 11q14.1-q14.3, caused by three GBMs: PGBS2051 (11q14), PGBS2126 (11q14-q22), UGBS3044 (11q14)), while a broader peak is at 11q23.2-11q24.3. Genes of interest include those for mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2; 11q13), vascular endothelial growth factor beta (VEGFB; 11q13), paired box 6 (PAX6; 11q13), cell adhesion molecule CD44 (CD44; 11q13), cyclin D (CCND; 11q13).

Losses in the 11p-arm: Two tumours lost genetic material at the centromeres, PGBS2532 (11p11.1-q13) and RGBC1724m (11p12-q11), with the latter extending for two bands above the heterochromatin region. These losses span the loci for cyclin D (CCND), vascular endothelial growth factor, beta (VEGFB) and mitogen-activated protein kinase kinase kinase kinase 2 (MAP4K2)(already mentioned). In addition, the gene for proteoglycan 2 and solute carrier 43 alpha, member 3 polypeptide, (PRG2andSLC43A3) that has structural similarities with bone morphogenetic protein 4, (BMP4) is mapped to 11q11. Two tumours, AS2706 and PGBS2532, were deleted at 11p15.2-11p15.5, which includes the loci for TFIIH1 and ASCL3 genes.

Losses in the 11q-arm: A common region lost in 3 tumours, GBS2126 (11q12), PGBS2532 (11p11-q13), and PGBS1595m (11q12-q13), is located in the proximal q-arm at 11q12.2-11q13.1. RGBC1724 and RGBC1724m are both deleted at 11q22.1-11q24.3. The common deleted regions span the loci for CCND, VEGFB, MAP4K2, PRG2andSLC43A3 and CTNND1 (11q12).



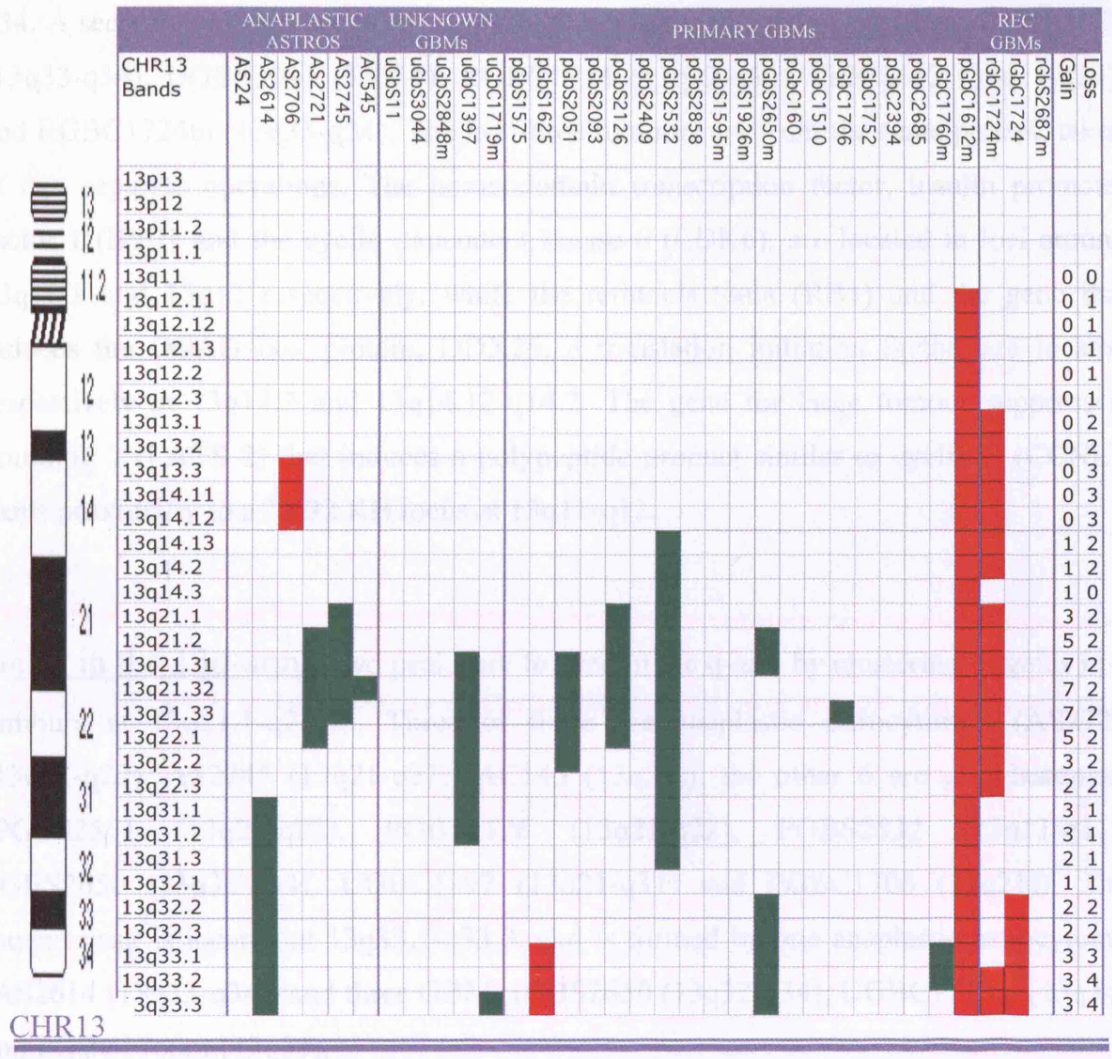
3-4-17 Figure 3-20 – CHR 12: Ideogram and Excel graph of CNAs for 12 HGAs.

12 tumours are informative for CNAs in this chromosome, all of them gaining with the exception of UGBS3044 (12q24.1-q24.3) and PGBS2126 (12q24.3), which were deleted in a corresponding region of the q-arm, making the 12q24.3 the only common region to be deleted in chromosome 12. A gene of interest in this region is that of general transcription factor 2H, polypeptide 3 (GTF2H3; 12q24.31)

<u>Gains in the 12p-arm:</u> Two common regions of gain occur, one at 12p13.32-12p13.33 caused by 4 tumours, AS2614 (12p12-p13) and three GBMs (GBS1926 (12p13), UGBC1719m (12p13), PGBC1760m (12p13), and a second at 12p11.p12,

arising from gains in two glioblastomas, PGBS2126 (12p11.1-p11), and GBS1595 (12p11.1-p12). The genes of interest in this region include those for the microsomal glutathione S-transferase 1 (MGST1; 12p12.3-p12.1), neurotrophin 3 (NTF3; 12p13), glyceraldehydes-3-phosphate dehydrogenase (GAPD; 12p13), cyclin dependent kinase 1B/p27/KIP1 (CDKN1B; 12p13.1) and neural enolase 2 gamma (ENO2; 12p13).

Gains in the 12q-arm: There is a major cluster of CNAs around the middle of the 12q-arm involving 8 tumours with a peak at 12q14.3-12q21.2. One of the tumours that gained is an astrocytoma, AS2745 (12q15-q31), and the others are GBMs, GBS1575 (12q13-q21), PGBS1625 (12q13-q15), PGBS2051 (12q15-q21), PGBS2126 (12q13.3-q21.32), PGBS2858 (12q14-q15), UGBS3044 (12q15-q21) and GBS1595 (12q14-q21). Several genes of interest are mapped to the listed 12q-arm regions, among them those for Cyclin dependent kinase 2 (CDK2; 12q13), cyclin dependent kinase 4 (CDK4, 12q14), the MDM2-transformed 3T3 cell double minute 2, a p53 binding protein (MDM2, 12q14.3-q15), BRCA1 associated protein (BRAP, 12q24) and mitogen-activated protein kinase-activated protein kinase 5 (MAPKAPK5, 12q24.12). One tumour, UGBC1719m (12q24.3) gained genetic material further down the chromosome, at bands 12q24.31-q24.32, which spans the 28.83 Kb locus of the general transcription factor 2 H, polypeptide 3 (GTF2H3, 12q24.31).



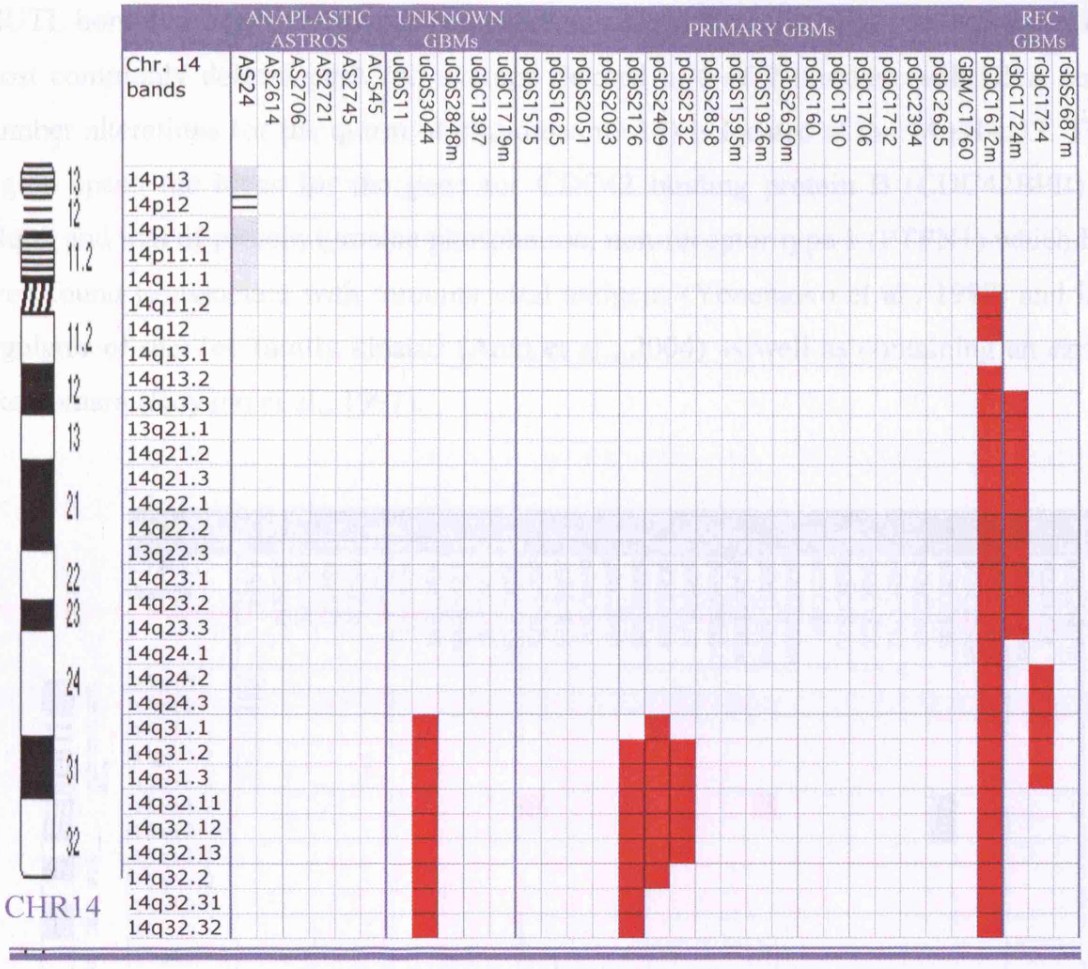
3-4-18 Figure 3-21 – CHR 13: Ideogram and Excel graph of CNAs in 17 HGAs

Seventeen tumours (69.7%) showed DNA copy number changes in CHR13, with 5 out of the only 6 anaplastic astrocytomas showing alterations in DNA copy number. Two tumours, PGBC1612 (13q11-q34, and RGBC1724 (13q13-q14; 13q21-q22; 13q33-q34) had extensive losses or loss of the entire q-arm while 15 others had alterations limited to a single band or moderate lengths.

Loss in the 13q-arm: Three tumours were jointly deleted around 13q13-q14 resulting in a peak at 13q13.2-q14.12 made by AS2706 (13q13-q14) and two related glioblastomas, GBC1612, which lost the entire q-arm (13q11-q34) and RGBC1724 (13q13-q14). RGBC1724 has other losses distal to this peak, at 13q21-q22 and 13q33-

q34. A second peak is situated at 13q33.1-q33.3 and is formed by 5 GBMs, PGBS1625 (13q33-q34), UGBS3044 (13q34), RGBC1724 (13q32-q34), GBC1612 (13q11-q34), and RGBC1724m (13q33-q34). The last three tumours are from the same patient, taken at two separate operations. The homeodomain transcription factor, insulin promoter factor 1 (IPF1) and the cyclin dependent kinase 6 (CDK6), are located in loci around 13q12.1 and 13q12 respectively, while the retinoblastoma (RB1) and the gene that induces the DEAD box protein, DDX26, a translation initiation factor, are located respectively at 13q14.2 and 13q14.12-q14.2. The gene for large tumour suppressor, homolog 2 (LATS 2) that induces a polypeptide product similar to cyclin E (CCNE), maps proximally to a 12.32 KB locus at 13q11-q12.

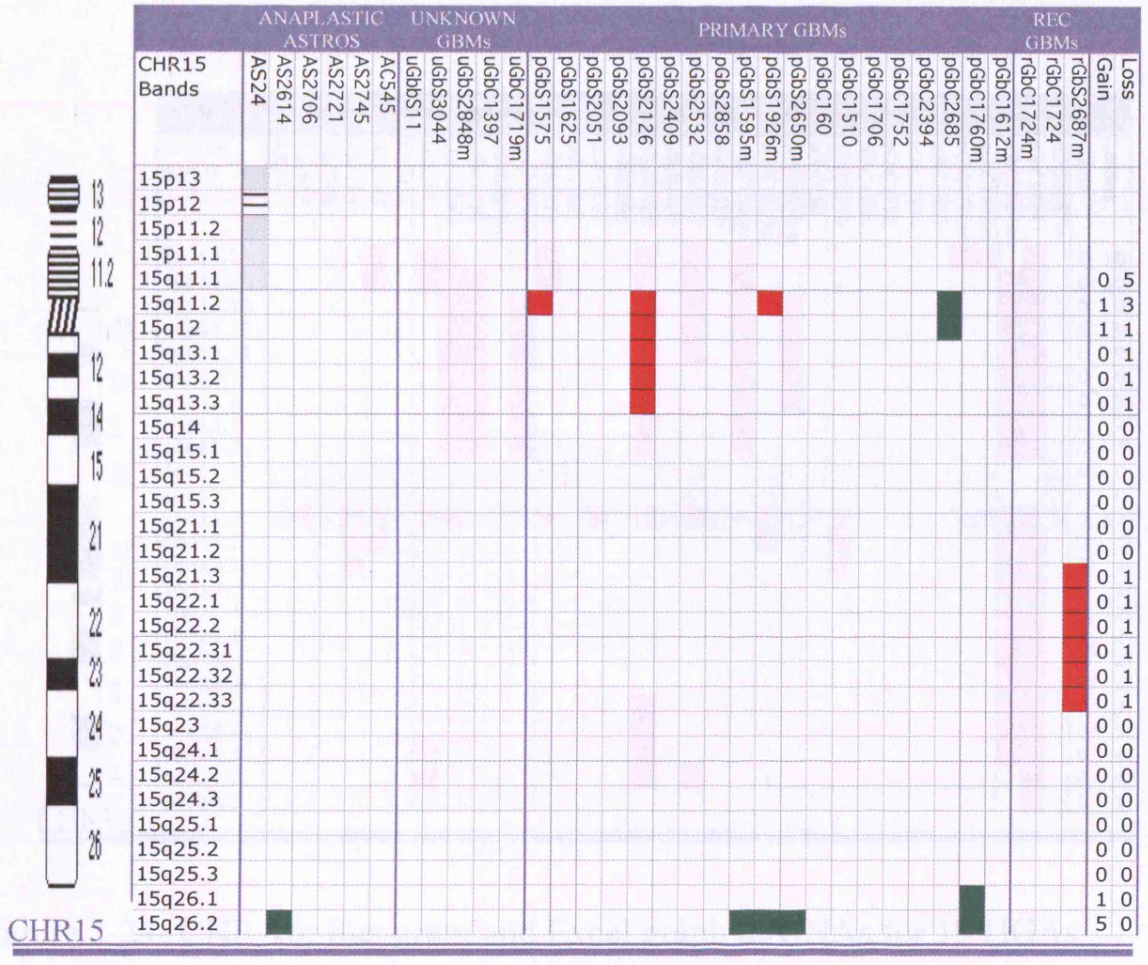
Gains in the 13q-arm: Two peaks are formed in the q-arm by clustering of gains in 9 tumours at 13q21.1-q21.33. Three of these are anaplastic astrocytomas (AS2721 (13q21-q22), AS2745 (13q21-q31), AC545 (13q21)), the other 6 are glioblastomas, (PGBS2501 (13q21-q22), PGBS2126 (13q21-q22), PGBS2532 (13q11-q31), PGBS2650 (13q32-q34), UGBC1397 (13q21-q31) and PGBC1706 (13q21)). The second peak is located at 13q33.1-q33.3, and is formed by one anaplastic astrocytoma (AS2614 (13q33-q34)) and three GBMs (GBS2650 (13q32-q34), UGBC1719m (13q34) and PGBC1760m (13q33).



3-4-19 Figure 3-22 – CHR 14: Ideogram and Excel graph of CNAs for 7 HGAs

Losses in the 14q-arm: One tumour, PGB1612 (14q11.1-q12; 14q13-q32), almost completely lost the entire q-arm, while the related tumour (RGBC1724m) lost a large segment (14q24-q31). Five other glioblastomas, PGBS2126 (14q31-q32), PGBS2409 (14q31-q32), PGBS2532 (14p13-q31), UGBS3044 (14q31-q32) and RGBC1724 (14q24-q31) have deletions that cluster around 14q24.2-q32.32, forming a peak at 14q31.2-q32.13, which includes PGBC2685. The genes of interest in the proximal and middle portions of the q-arm include those for thyroid nuclear factor / thyroid transcription factor 1 (TIF1 (47.01 Kb)) and NK2 transcription factor related, locus 9 (NKX2-9 (2.58 Kb)), both located at 14q13.3, mitogen-activated protein kinase kinase kinase kinase 5 (MAP4K5) at 14q14.2-q21, bone morphogenetic protein 5 (BMP5/BMP5.1) at 14q23-q24, V-AKT/AKT1, the human homolog of the murine thymoma viral oncogene, which is located at 14q23.32 and the mismatch repair gene

MUTL homolog 3 (MLH3) which is located at a 37.74 Kb locus at 14q24. However, the most commonly deleted peak (on average deleted in 6 of 7 tumours with DNA copy number alterations for the q-arm of chromosome 14) is located at the 14q31-q32. This region spans the locus for the gene for CDC42 binding protein B (CDC42BPB) at 14q32 and that of protein tyrosine phosphatase, non-receptor type 1 (PTPN1) which has been found to associate with sarcoma viral antigens (Yonemoko et al., 1987) and is a regulator of the Tec family kinases (Aoki et al., 2004) as well as containing an ezrin-like domain (Koyano et al., 1997).



3-4-20 Figure 3-23 – CHR 15: Ideogram & Excel graph of CNAs for 11 HGAs

<u>The 15q-arm CNAs:</u> 9 tumours had aberrations in the q-arm of CHR15. One tumour, RGBS2687m lost material in the middle of the q-arm at 15q21.2-q22.32, spanning the

79.79 Kb locus of the gene for mothers against decapentaplegic homolog 6 (SMAD6), located at 15q21-q23. The 15q26 region is deleted in 4 tumours – one an anaplastic astrocytoma, AS2614 (15p26), and 3 GBMs, PGBS1926m (15q26), RGBS2687m (15q21-q23), PGBS1760m (15q26) and GBS2650 (15q26). The neogenin homolog 1 (NEO1), which has homology with deleted in colon cancer (DCC.1), and could be implicated in familial gliomas (Latil et al., 2003), is mapped to a 2562.65 Kb locus at15q22.3-q23 (Vielmetter et al., 1997). Several genes of interest (GOIs) are mapped to the most commonly gained region of chromosome 15 among them, the feline sarcoma viral homolog (FES; 15q26.1) and the mesodermal posterior 1 (MESP1) also at 15q26.1

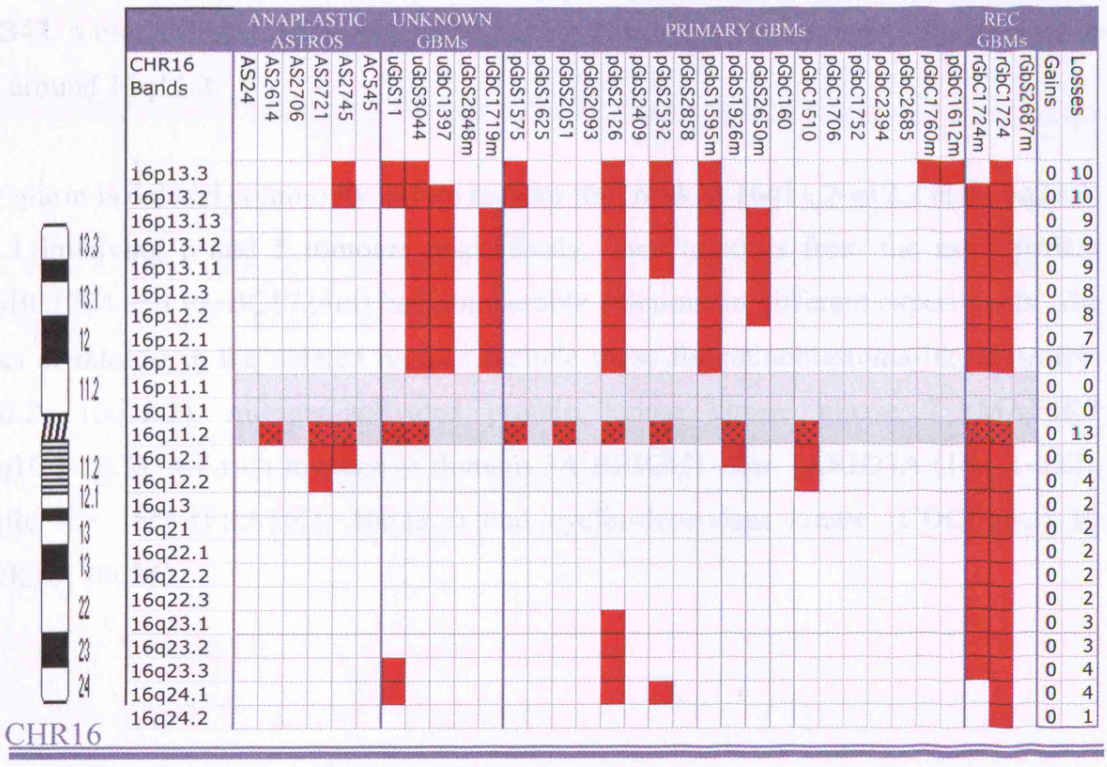


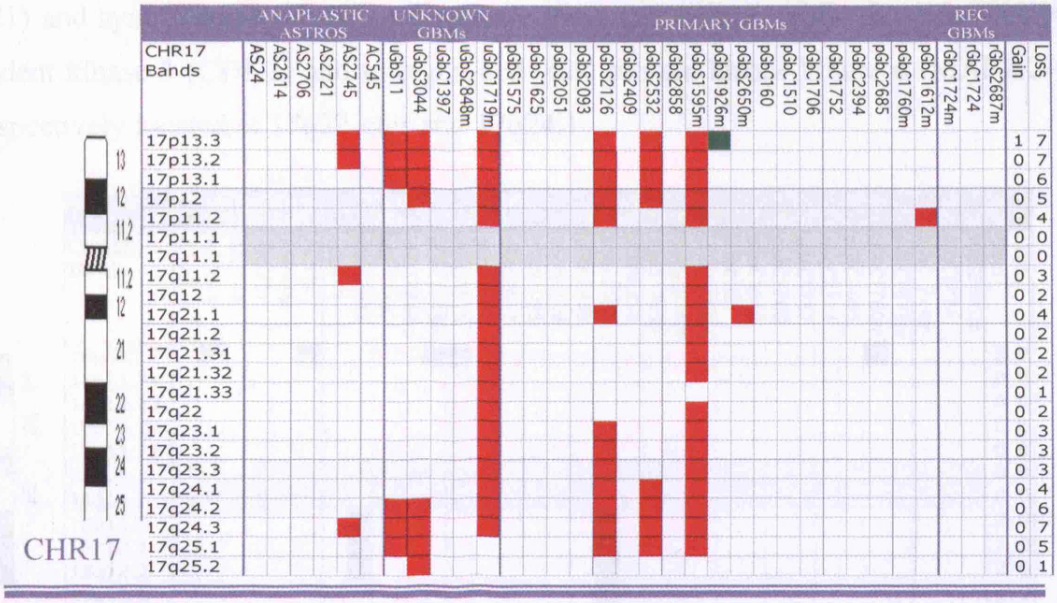
Figure 3-24- CHR 16: Ideogram and Excel graph of CNAs for 19 HGAs

Nineteen tumours showed DNA copy number changes in this chromosome, and all of them were deleted predominantly in the p-arm and at the pericentromeric heterochromatin (in the proximal q-arm). Two peaks can be made out in the p-arm, one located at 16p13.2-p13.3, involves 12 tumours, while the second one, with a broader base, is at 16p12.3-p13.12 and includes 9 tumours. The 34.52 Kb and 65.33 Kb loci of the genes for mitochondrial ribosomal protein L28 (MRPL28), axin1 (AXIN1) protein,

which functions as a negative regulator of the wingless-type MMTV integration site family, member 1 (WNT) signaling pathway (Salahshor and Woodget, 2005) and can induce apoptosis (Rui et al., 2004); and tuberous sclerosis type 2 (TSC2), are all mapped to loci around 16p13.1-13.3

16 tumours, 3 of them AAs are deleted in the centromere and noncentromeric heterochromatin region where a sharp peak is evident at 16p11.1-q11.2. The 16p11.1 band is involved in 8 tumours while the 16q11.2 is involved in 13 (38.3%). The gene for myosin light chain kinase (MLCK; 16q11.2), which is involved in Ca2+/CaM mediated (Fajmut et al., 2005) and ATP-mediated P2X1-dependent ERK2 pathways (Mongin and Kimelbergh, 2005), and LOC440374, the gene encoding Similar to RAB43, a member of the Ras oncogene family (16q11.2), are among GOIs mapped to loci around 16q11.2

The q-arm is deleted commonly in two regions that peak at 16q11.2-q12.2 and 16q23.3-q24.1 involving 6 and 5 tumours respectively. Two tumours from the same patient (RGBC1724 and RGBC1724m) had comparable deletions in different experiments. The genes of interest in the deleted regions include those for retinoblastoma-like 2 protein (RBL2; 16q12.2), mitogen-activated protein kinase kinase kinase 7 (MAP3K7 (16q16.1-16.3), AT rich interactive domain 3A BRIGHT-like (ARID3A (16q21-q22), fragile site 16D (FRA16D; 16q23.3) and cyclin-dependent kinase (CDC2-like) 10 (CDK10; 16q24).



3-4-21 Figure 3-25 – CHR 17: Ideogram & Excel graph of CNAs for 10 HGAs

Ten tumours were altered in this chromosome, with only one, PGBS1926m (1p13.3), showing a gain. Nine tumours were deleted in the p-arm, forming a peak a broad-based peak at 17p12-p13.3 in which 7 of the 9 tumours are involved. 9 tumours were deleted in the q-arm, 4 of them at 17q21.1 while 6 were jointly deleted at 17q24.1-q25.1. Three tumours, PGBS2126 (17p11.1-p13), PGB1595 (17p11.2-q21) and UGBC1719m (17p11.2-p13) lost the entire p-arm as well as large sections or the entire q-arm. Genes of interest at 17p13 are TP53 that encodes tumour protein p53, located at 17p13.1, transcription regulator eukaryotic initiation factor 4, A1 (EIF4A1), which is located at 17p13 and two genes with roles in neurogenesis: hairy/enhancer of split, homolog 7 (HES7) and neurogenic differentiation factor 2 (NEUROD2) both located in loci at 17p13.1.

The deleted regions in the q-arm harbour several GOIs among them, the v-erb-b erythroblastic leukaemia viral oncogene homolog 2 (ERBB2), which is located at 17q11.2-q12 and the genes for glial fibrillary acidic protein (GFAP), breast cancer 1, early onset (BRCA1), interferon induced protein 35 (IFI35), keratin 25 C (17q21.2), and all 11 homeobox class B genes (HOXB; 17q21.3-q22), are mapped in loci clustered around the 17q21-q22 chromosome region. The noggin-related symphalangism 1

(SYM1) and synostoses 1 (SYNS1) genes are located at 17q22, while those for cyclin dependent kinase 3 (CDK3) and mitogen-activated protein kinase kinase 6 (MAP2K6) are respectively located at 17q22-qter and 17q24.1

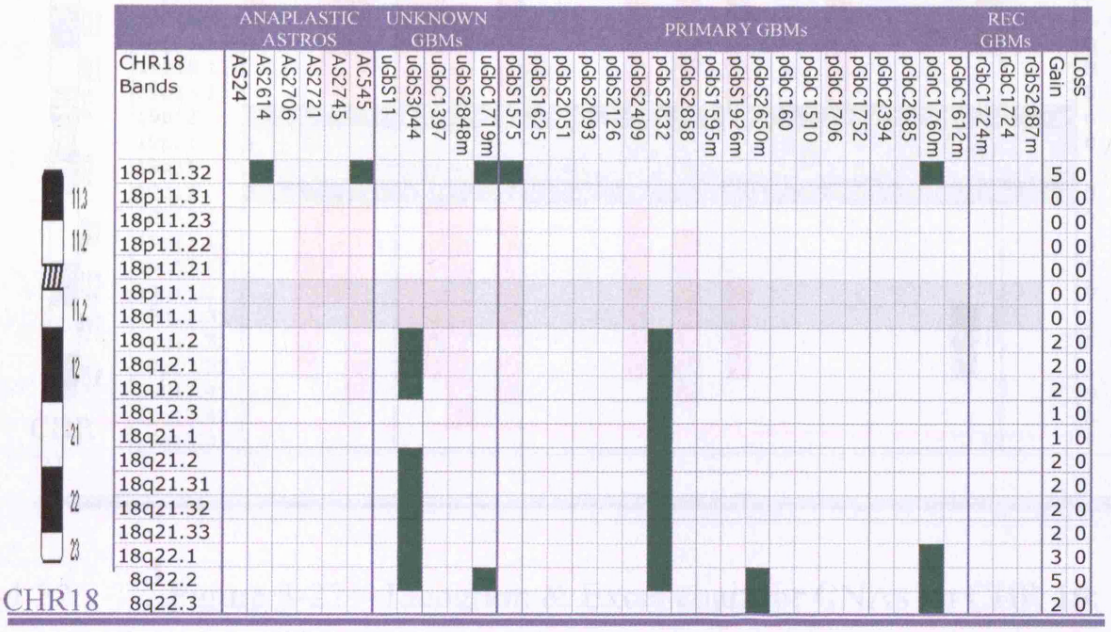
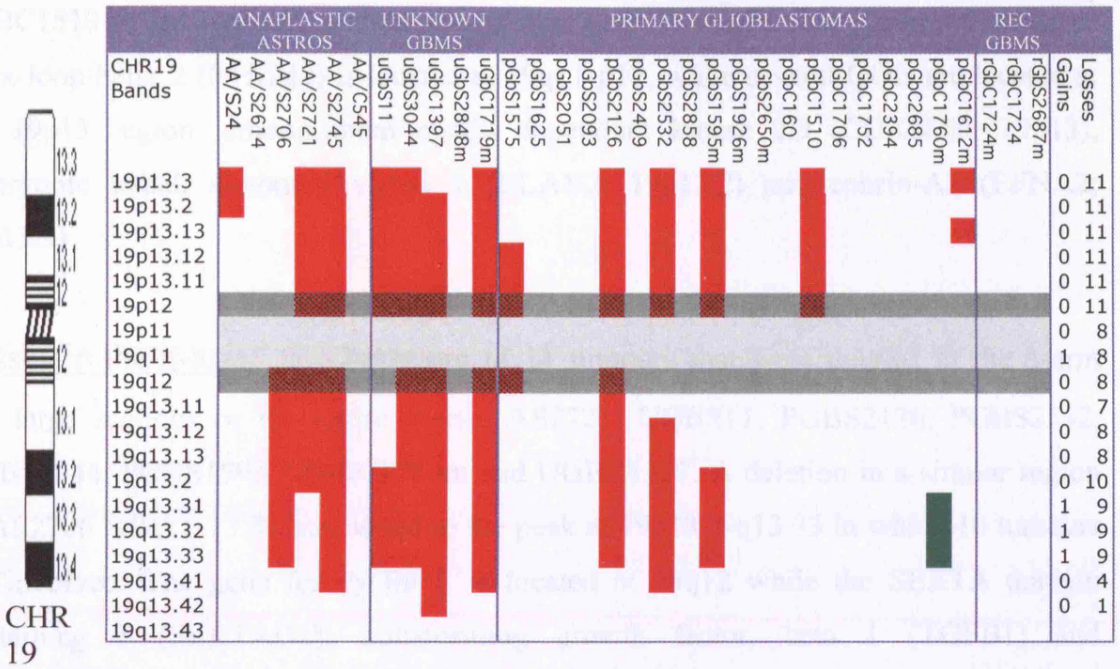


Figure 3-26 – CHR 18: Ideogram and Excel graph of CNAs from 8 HGAs

Eight tumours were informative for copy number aberrations on chromosome 18 and all were gains involving the telomere ends of the p-arm in 5 tumours: 2 anaplastic astrocytomas, AS2614 (18p11.3), AC545 (18p11.3) and 3 GBMs, PGBS1575 (18p11.3), UGBC1719m (18p11.3) GBC1760 (18p11.3), making 18p11.3 the only band gained in the p-arm. The ribonucleic acid (RNA) guanine-7-methyltransferase gene is located at 18p11.22-p11.23

Six tumours gained genetic material in the q-arm, PGBS2093 (18q23), PGBS2532 (18q11.2-q22), UGBS3044 (18q11.2-q12; 18q21.2-q22.2), GBS2650 (18q22-q23), UGBC1719m (18q22-q23) and PGBC1760m (18q22-q23). Two of them, PGBS2532 and UGBS3044 gained nearly the entire q-arm. A peak was formed at chromosome band 18q22.2 in which 5 out of six tumours with DNA copy number alterations were involved. The 30.86 Kb locus of the gene for mothers against decapentaplegic (DPP) homolog 7 (SMAD7) is located at 18q21.1, while the deleted in colon cancer genes (DCC and DCC.1) are mapped to loci at 18q21.3.



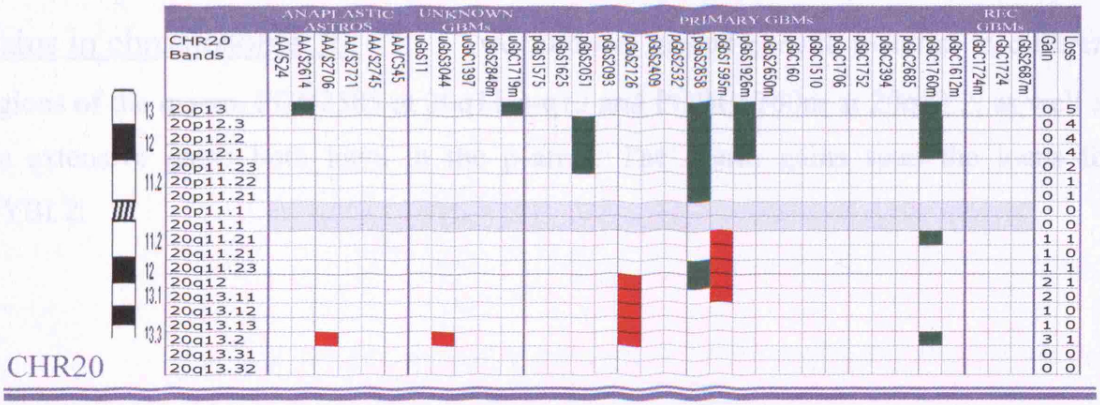
3-4-22 Figure 3-27 – Ideogram & Excel chart for CNAs on CHR 19:

Fifteen tumours showed DNA copy number changes in this chromosome, which was almost exclusively deleted, to a similar extent in both arms, except for the gains in the q-arm of GBC1760, at 19q11 and 19q13.31-q13.33. Three anaplastic astrocytomas lost the entire arm or large portions of both the p- and q-arms: AS2706 (19q11-q13.3), AS2721 (19p13.3-q13.2), AS2745 (19p13.3-q13.4)), as did 7 glioblastomas, UGBS11 (19p12-p13.3; 19q13.2-q13.3), PGBS2126 (19p13.3-q13.43), PGBS2532 (19p13.3-q11; 19q13.11-q13.41), UGBS3044 (19p13.3-q13.3), PGBS1595 (19p11-p13.3; 19q13.2-q13.3), UGBC1397 (19p13.3-q13.4), PGBC1510 (19p12-p13.3) and UGBC1719m (19p12-p13.3; 19q12-q13.3).

<u>Pattern of deletions in the p-arm:</u> Ten of the 13 tumours showing deletions of this arm lost the entire arm or major portions of it. However, the loss slightly peaked at 19p13.2-p19.3 where three tumours, AS24 (19p13.2-p13.3), PGBC1510 (19p12-p13.3) and GBC1612 (19p13.2; 19p13.3)) were deleted similarly. Eleven of 15 tumours with DNA copy number alterations lost significantly larger sections or entire p-arms: AS2721 (19p13.3-q13.2), AS2745 (19p13.3-q13.4), UGBS11 (19p12-p13.3), PGBS1575 (19p13.1-q12), PGBS2126 (19p13.3-q13.43), PGBS2532 (19p13.3-q11), UGBS3044 (19p13.3-q13.3), PGBS1595 (19p11-p13.3), UGBC1397 (19p13.3-q13),

PGBC1510 (19p12-p13.3) and UGBC1719m (19p12-p13.3). The gene for nascient helix-loop-helix 2 (NHLH2) is located at 19q11-p12, while several GOIs are located at the 19p13 region, among them cyclin dependent kinase 2D (CDKN2D; 19p13), embryonic lethal, abnormal vision 2 (ELAV2; 19p13.2) and ephrin-A2 (EFNA2; 19p13.3)

Losses in the q-arm: Eight out of 11 tumours that were deleted in the q-arm lost large sections or the entire q-arm: AS2721, UGBS11, PGBS2126, PGBS2532, UGBS3044, PGBS1595, UGBC1719m and UGBC1397. A deletion in a similar region of AS2706 (19q11-13.3), has added to the peak at 19q13.2-q13.33 in which 10 tumours are involved. The gene for cyclin E is located at 19q12 while the SERTA domain containing 1 (SERTAD1), transforming growth factor, beta 1 (TGFB1) and neurotrophin 5 (NTF5) are located respectively at loci around 19q13.1-q13.2, 19q13.2 and 19q13.3



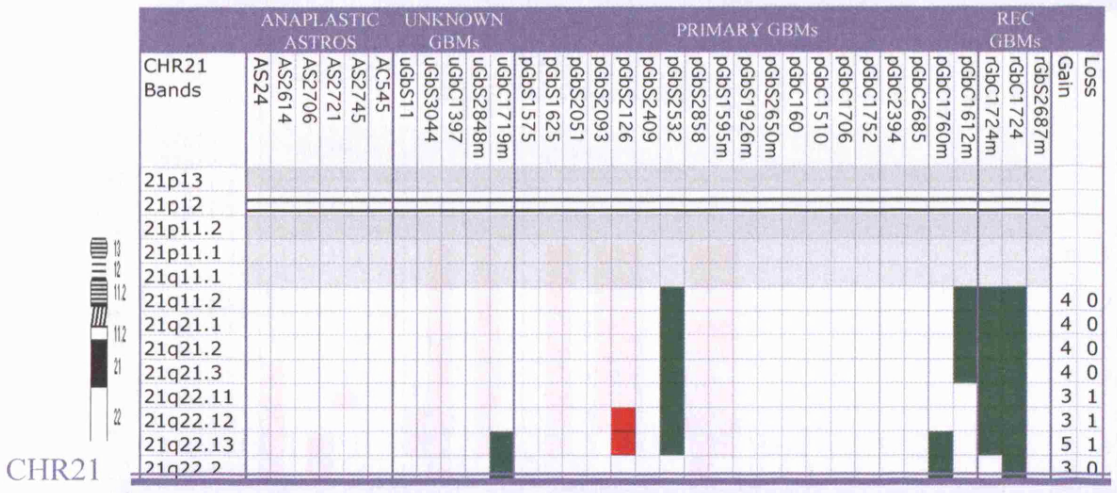
3-4-23 Figure 3-28 – CHR 20: Ideogram & Excel graph of CNAs for 10 HGAs

Ten tumours, one of which is an anaplastic astrocytoma, are informative for copy number aberrations on chromosome 20. Of these, six have CNAs in the P-arm. The six tumours, AS2614 (20p13), PGBS2051 (20p11.2-p12), PGBS2858 (20p11.1-p13), PGBS1926m (20p12-p13), UGBC1719m (20p13), and PGBC1760m (20p12-p13; 20p11.2-q11.2), all showed gains which form two peaks, one at 20p12.1-p12.3 and another at 20p13.

Several GOIs map to loci around 20p-arm. These include the neurotrophin factor 2 (NFT2) – like export factor 1 (NXT1) and DNA-binding and chromodomain-regulatory gene, SRY-BOX 12 (SOX12/SOX20; 20pter-q11.23) at 20p12-p11.2, and the genes for NK2 transcription factor related, locus 2 (NKXX2.2), cell division cycle 25B and chromosome 20 open reading frame 29 (CDC25BandC20orf29), and SMOX, a hypothetical gene which codes for polyamide oxidases (Wang et al., 2003) that may have roles in cell proliferation and survival (Murray-Stewart et al., 2002), map to respective loci of 3.14 Kb, 38.32 Kb and 68.81 Kb, all located around 20p13.

Six tumours are informative for CNAs in the q-arm, four are deleted, three of them at band 20q13.2 while two others share a broader deletion spanning the region 20q12-q13.11. The genes for E2F transcription factor (E2F; 20q11.2), chromodomain DNA binding protein 6 (CHD6/CHD5; 20q12), breast carcinoma amplified sequence 4 (BCAS4; 20q13.1) and v-myb, the myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2; 20q13.1), map to respective loci in the q-arm.

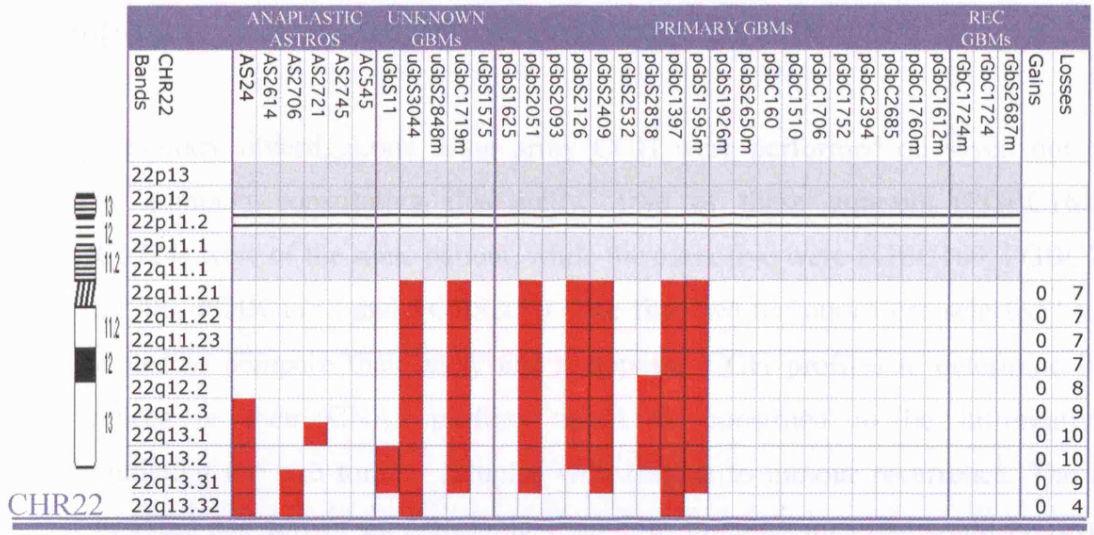
Gains in chromosome 20: Two tumours gained genetic material in different regions of the q-arm, PGB2585 at 20q11.3-q12 and PGBC1760m at 20q13.2, as well as the extensive gains both have in the p-arms. The q-arm gains span the locus for MYBL2.



3-4-24 Figure 3-29 – CHR 21: Ideogram and Excel graph of CNAs for 7 HGAs

Seven tumours showed DNA copy number changes in this chromosome, but most were clustered within the region of the acrocentromere (data not shown). 7 tumours had CNAs in the euchromatin region of the q-arm, of which only one (PGBS2126; 21q22.12-q22.13) was a deletion. Three tumours gained throughout most of the q-arm, PGBS2532 (21p11.2-q22), RGBC1724 (21p11.1-q22), RGBC1724m (21p11.2-q22) and formed two peaks at 21q11.2-q21.2 and at 21q22.13-q22.2, which involved three other tumours, GBC1612 (21q11.2-q21), UGBC1719m (21q22), GBM1760m (21q22). The S100 calcium binding protein, beta (neural) (S-100b) is mapped to 21q12.2.

The genes for oligodendrocyte lineage transcription factor 2 (OLIG2) and glutamate receptor, ionotrophic, kinase 1 (GRIK1) are located at loci around 21q22.1, while loci for several genes of interest, e.g., the genes for H2B histone family, member S (H2B), SH3 domain binding glutamic acid rich protein (WRDandSH3BGR) and runt-related transcription factor 1 (acute myeloid leukaemia 1 (aml1 oncogene (RUNX1 and RUNX1.1) are located at 21q22.3.



3-4-25 Figure 3-30 – CHR 22: Ideogram & Excel graph of CNAs for 12 HGAs

Twelve tumours were informative of CNAs in CHR22. The distribution of CNAs created two peaks, one, with a very broad base at 22q11.21-22q12.1, was formed by 7 tumours each of which lost large or complete stretches of the q-arm. A distal peak is located at 22q12.3-22q13.32 and is made up of losses from all 12 tumours with DNA copy number alterations. The genes for neurofibromin (bilateral acoustic neuroma; 22q12.2), hepatocyte growth factor-like (MGC17330; 22q12.2), kringle containing transmembrane protein 1 (KREMEN1; 22q12.1), that encodes receptors which cooperate in blocking WNT/catenin-signaling pathways, LIM kinase 2 (LIMK2; 22q12), checkpoint homolog 2 (CHEK2; 22q11) and Chromosome 22 breakpoint cluster region (BCR; 22q11), are among the GOIs mapped to respective loci in the q-arm.

Chapter 4: Results of Array CGH experiments

Supplementary investigations using array CGH were performed on seven out of 32 (~21%) tumours constituting this study. Two of these tumours (PGBC1612 & RGBC1724) were of the same patient, while the other five were PGBC160, PGBC1510, PGBC1706, PGBC1752 and PGBS2093. The first two tumours were selected because we wanted to compare their array and metaphase CGH profiles to determine if any differences in their CNAs profiles might be construed to be informative in differentiating the two tumour samples with respect to tumour recurrence. The other four tumours had few or no detectable alterations in autosomes that could be revealed by metaphase CGH. This factor made these tumours suitable candidates for further investigation using array CGH, to determine if there might be subtle copy number alterations below the resolution of metaphase CGH, that could account for the advanced histologically grade of the respective tumours.

This chapter of the thesis discusses the results of these 7 array CGH experiments for the purpose of gaining additional information from these tumours. Together with Chapter 5 (results of the MFISH experiment), which follows, this chapter also serves to illustrate the diagnostic potentials of various in-situ hybridisation (ISH) techniques, thus highlighting some of the experimental procedures that could form part of methodologies needed to carry forward some of the results obtained from the metaphase CGH experiments, in the hope that information gained from such studies could improve understanding of the oncopathogenesis of astrocytomas.

Array CGH experiments were performed using 1-mega-base (Mb) genomic chips produced at the Sanger Centre Cambridge, UK and the same DNA [taken from a normal male (ECACC Number: CBF 0008 HD03/01/176]) was used as a control in all microarray experiments.

The array CGH results of these seven tumours are shown alongside metaphase images in figures 4 (1-7). The results are directly produced respectively by the metaphase-CGH, and microarray CGH-analysis software. The metaphase and array data of tumours 1612 and 1724, which are from the same patient, are discussed in detail for each

chromosome, in sections 4, 7_1 (CHR1) to 7_24 (CHR-Y). Associated tables containing detailed genetic information on some of the outlying clones in all seven tumours, including the comparison of array and metaphase CGH for PGBC1612 and RDBC1724, are presented in Appendix 4. While these tables are referred to in the text, to avoid monotony, with a few exceptions no further reference to the appendix is made.

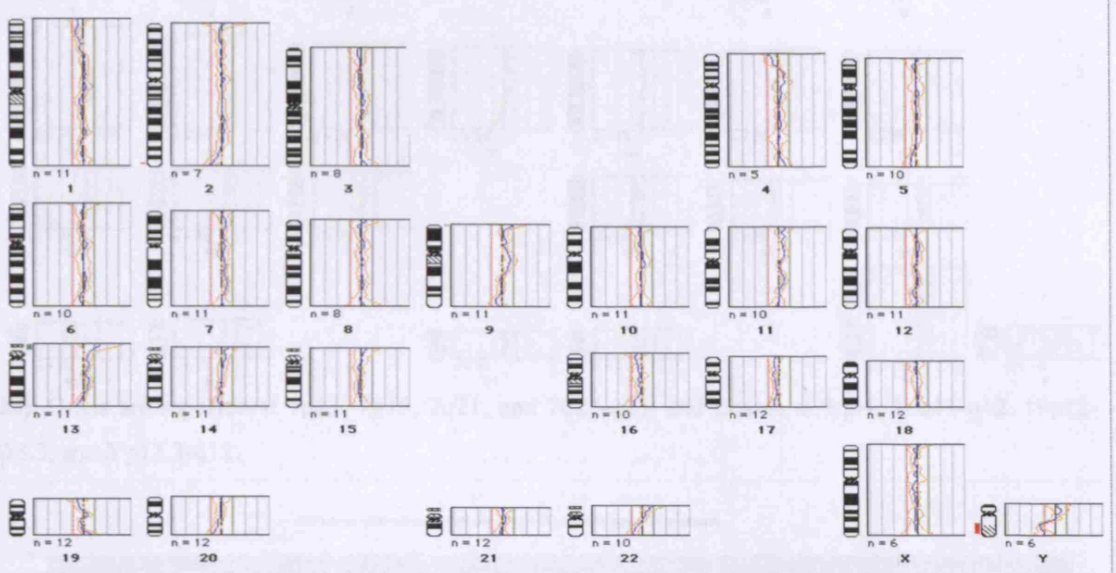
The normal range of fluorescence ratios between *test* and *normal* (also known as *reference*) DNAs, which is the reference point for interpreting outcomes of both metaphase and array-CGH experiments, has been taken as 1.0 +/- 0.2. The software for interpretation of the metaphase experiments generates graphs, which demonstrate their limits directly. Although the fluorescence intensities are presented in a standard numerical format, in the microarray Excel charts (data not shown on account of enormous size) the graphs of fluorescence intensities are generated on a log2 ratio. To convert fluorescence values represented in log2 to normal numerical equivalents, the following formula is applied: $log_y (y^x) = x$. "y" is the base of the logarithm, and x represents any value of fluorescence ratio in logarithm to base y (log_y). For example, the normal numerical equivalent of the fluorescence ratio, which appears as 0.5 in log2, = log2 ($2^{0.5}$) = 1.41 while that of the log2 on the negative side of the Y-ordinate, (i.e., log2 of -0.5) is ~0.71 (actual value, 0.7070).

4-1 Polymorphic and "problem" clones

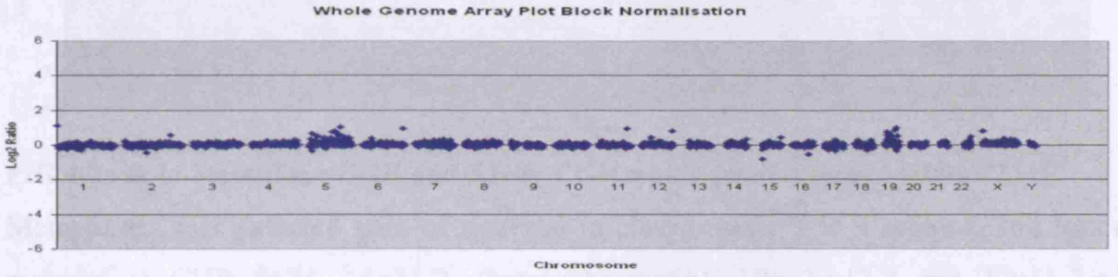
In interpreting data generated by the arrays, consideration was taken of known polymorphic (those that were previously shown to be common non-pathogenic polymorphisms) and problem clones (clones with sequences matching those of chromosomes other than the ones which the clone is primarily associated) are listed in Appendix 5

4-2 Metaphase and array CGH data

4-2-1 Tumour GBM/C160



(1a): The gain reported at 13p is in the heterochromatin region; Losses (Red) are reported at 2q37 and Yq12.

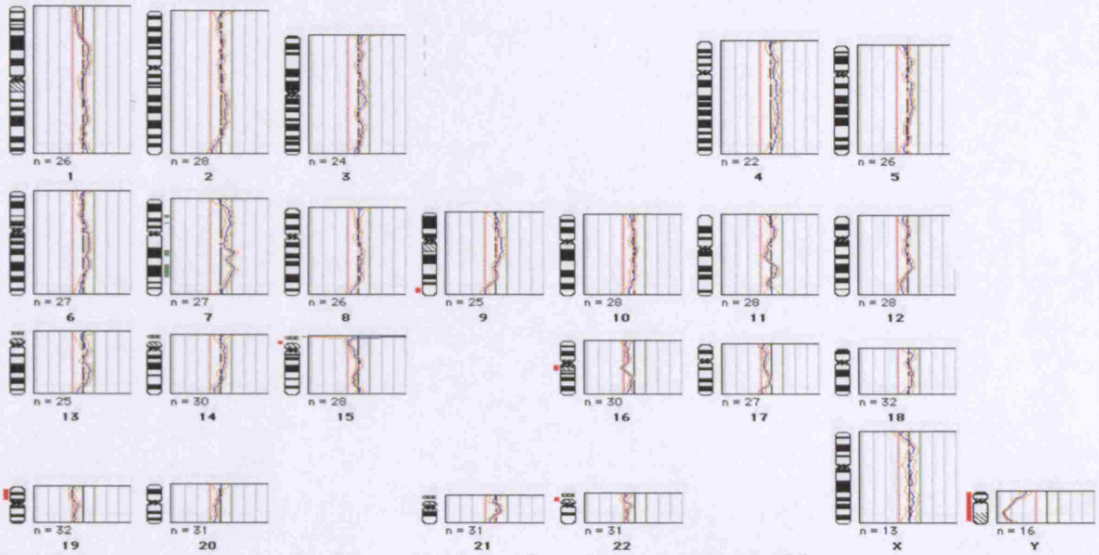


(1b)

Figure 4-1a & b: Metaphase and array CGH images of GBM/C160

Metaphase CGH revealed one apparent CNA, a deletion at 2q37, while the rest of the genome was apparently normal. The whole genome array-CGH plot shows the majority of the clones have normal Test/Ref DNA ratios, which validates the metaphase experiment. However, the array plot revealed several clusters of clones that gained material in CHRs 5 and 19, and a number of scattered ones on chromosomes 1p, 2q, 6q, 11q, 12q and Xp. One clone is underrepresented in CHR 15q and another in CHR 16q. Detailed information on the altered clones, sourced from the NCBI Clone Registry database (www.ncbi.nlm.nih.gov/genome/clone/), is presented in Appendix 4.

4-2-2 Tumour GBM/C1510



(2a): Gains are reported at 7p13, 7p15, 7q21, and 7q22-q23; and Losses at 9q34, 16q11-q12, 19p12-p13.3, and Yp13.3-q12.

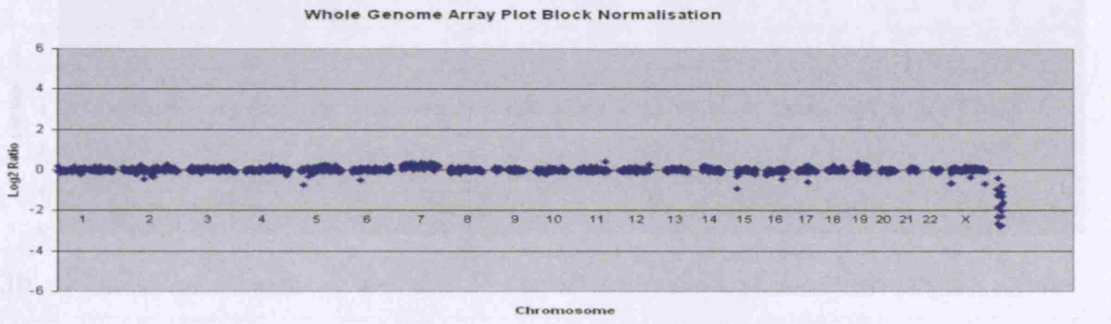
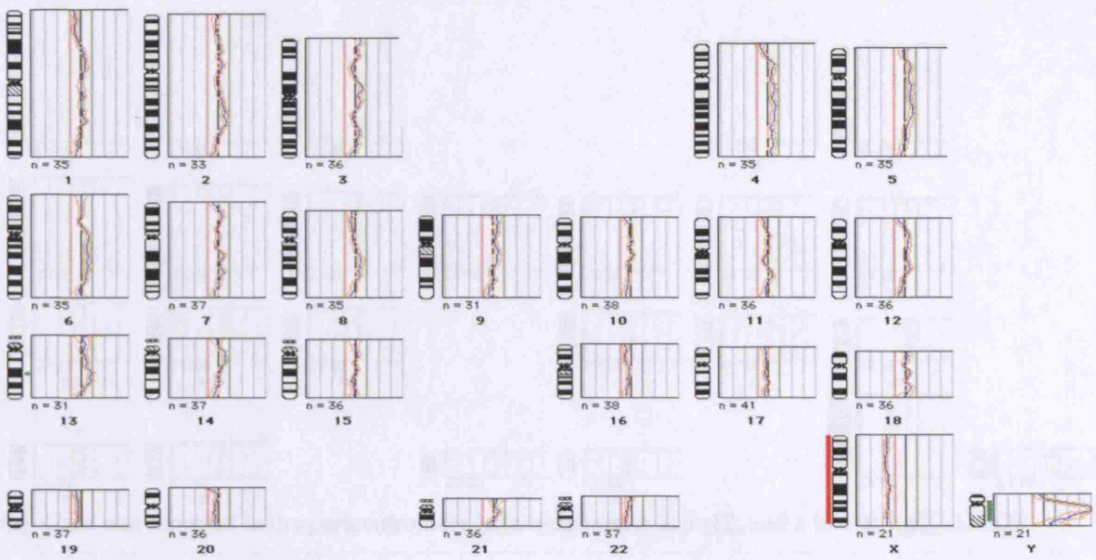


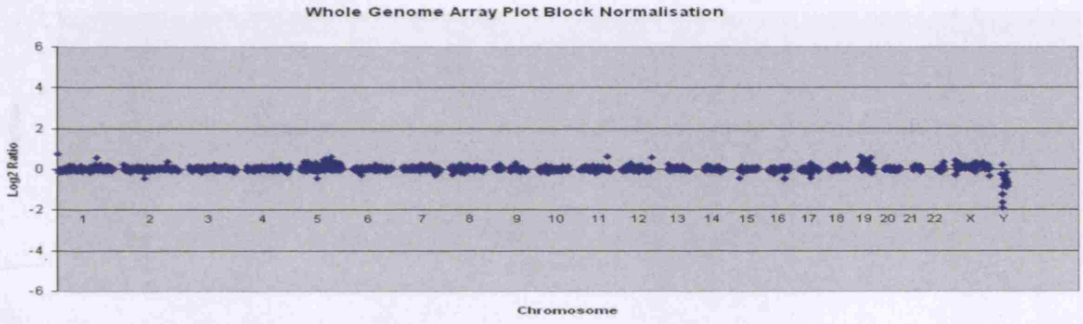
FIG 4-2a & b: Metaphase CGH and Array CGH whole genome plot of GBM/C1510.

Metaphase CGH detected gain of material in chromosome 7 in 4 regions, and loss of material at CHR 9q34, 16q11.2, (heterochromatin), 19p12-p13.2 and 22p11.2-p12 (heterochromatin). CHR 15p (Heterochromatin) was underrepresented both on metaphase and microarray. The remainder of the genome was essentially normal. The loss on 19p, reported in the metaphase is not evident in the microarray. The whole of CHR 7 is slightly overrepresented, which is also in agreement with the graph of average fluorescence ratios on metaphase chart. A number of scattered clones were deleted at 2p, 5p, 6p, 15, 16q, and 17. One clone each showed gain of material on 11q and 12q. in Appendix 4, shows data on genes located in regions that are altered in the array CGH plots.

4-2-3 Tumour GBM/C1706



(3a): Gains were reported at 13q21 and Yp13.3-q12; and Losses at Xp22-q28



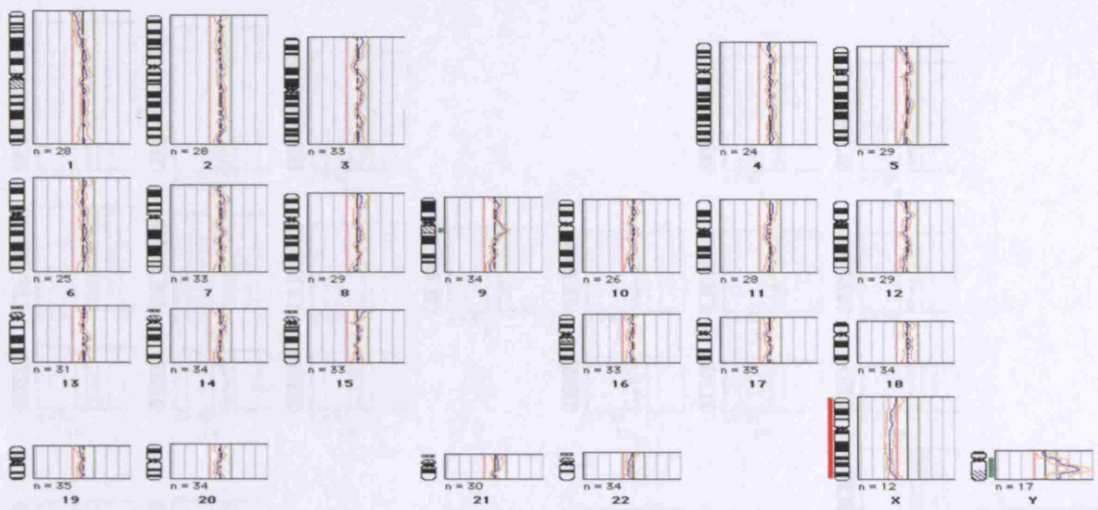
3b

Figure 4-3a & b: Metaphase and array CGH data for GBM/C1706

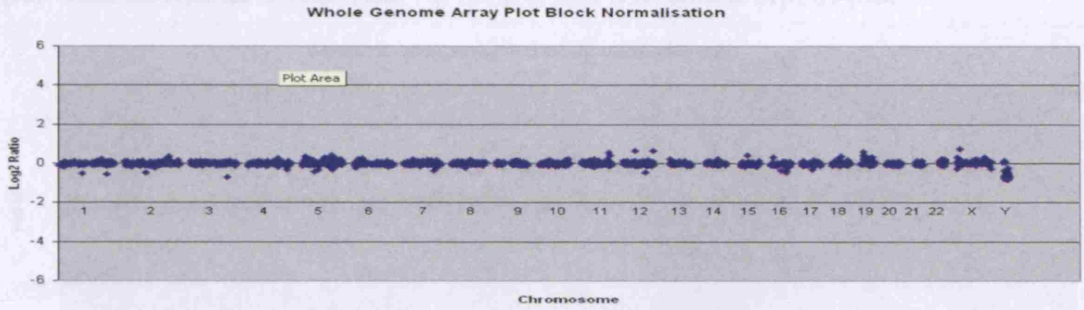
The two graphs revealed contradictory profiles, which is evident both in the X- and the Y-CHRs. Metaphase CGH showed copy number alterations at two regions: a gain at 13q21 and loss of the whole X-chromosome (Xp22.1-q28). The loss reported in the X-chromosome may represent an experimental artifact. It is likely due to differences in gender origins of reference DNA used in hybridized probe. GBM/C1706 was from a male patient, however, female reference DNA was used in the metaphase CGH experiment, which could explain the apparent gains in the Y-CHR.

Array CGH mostly showed a normal genomic profile. CHRs 5 and 19 were slightly overrepresented in some regions. A sample list of the most altered clones is presented in Appendix 4.

4-2-4 Tumour GBM/C1752



(4a): Gain was reported in the pericentromere heterochromatin at 9q12, and a loss at Xp22.33-q28

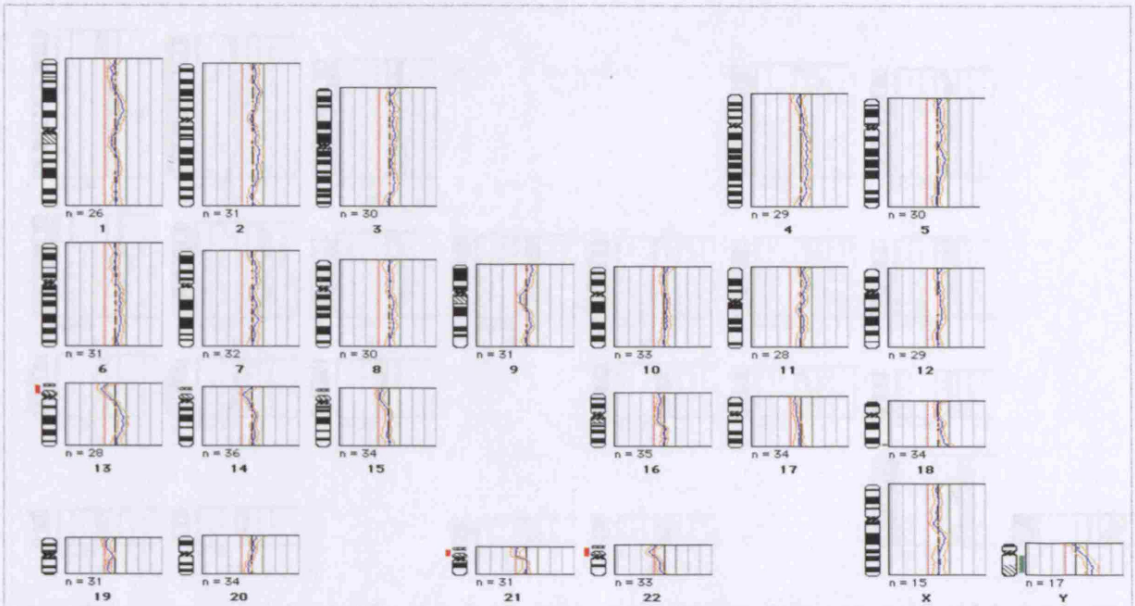


4b

Figure 4-4a & b: Metaphase and Array CGH for GBM/C1752

Metaphase CGH of GBM/C1752 showed a gain at 9p12, a known polymorphic region, and may reflect normal variation. The loss of genetic material on CHR X and gain on Y are likely due to gender differences in the source of DNAs used in the hybridization probe. The whole genome array plot shows the genome as mostly normal with a few scattered clones having gained on CHRs 11, 12, 15, 16 and 19, while several were deleted on CHRs 1, 2, 3, 12 and 16. Data on some of the altered clones are presented in Appendix 4.

4-2-5 Tumour GBM/S2093



(5a): Gains are reported at 18q23, and Yq11.1-q12; Loss is reported at 21p11.1-p13.

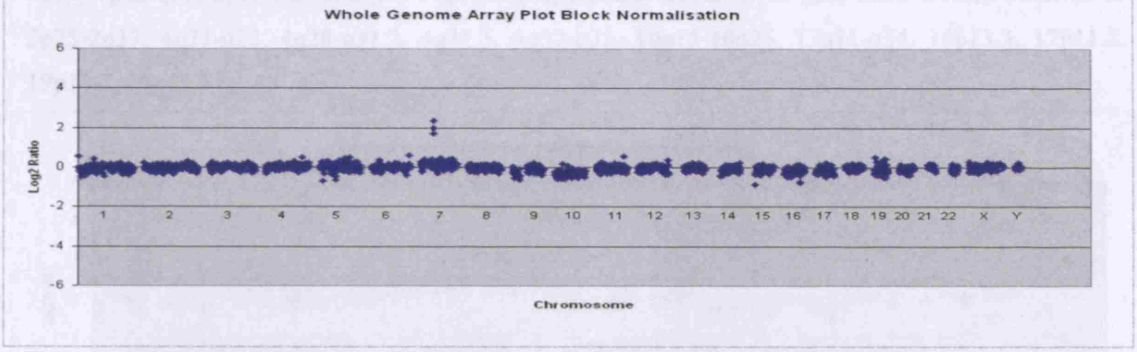
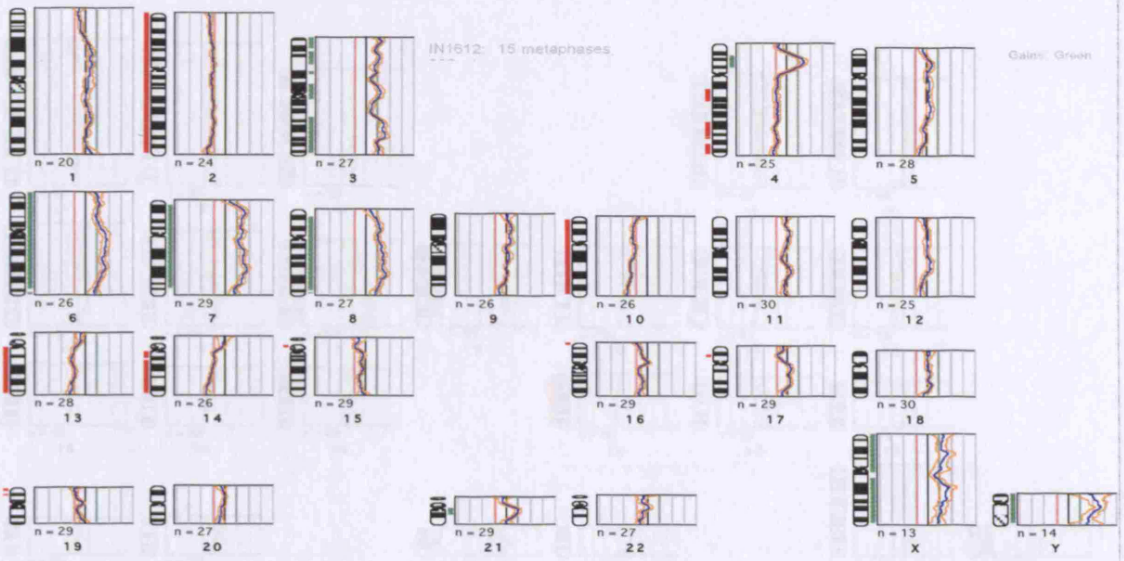


Figure 4-5a & b: Metaphase and Array CGH data for GBM/S2093

Metaphase CGH did not reveal a significant copy number alteration in this tumour sample; all those revealed are in heterochromatin regions, which are characterized by highly repetitive DNA sequences. The whole chromosome plot showed most of the genome as normal. There were 3 significantly overrepresented clones on CHR 7p while elsewhere in the genome a few showed gains or losses that were close to the cutoff limits for reporting CNAs. Two of the three contiguous clones that are overrepresented in 7p, RP11-1091E12 (at 54.85 MB CHR position) and RP11-339F13 (at the 55.01 MB CHR-position) both contain EGFR sequences. The EGFR is the most commonly amplified gene in high malignancy grade astrocytic tumours, thus gain of EGFR would confirm that the original tissue sample contained tumour material. Details of some of the most altered clones are presented in Appendix 4.

4-2-6 Tumour GBM/C1612



(6a) Gains were reported at 3p12-q11.2, 3p14, 3p21-p24, 3p24-p26, 3q21-q26, 4p13-p15.2, 6p25-q25, 6q27, 7p22-q35, 8p22-q24.2, 8q24, 21q11.2-q21, Xp22.3-Xq12, Xq13-q28; while Losses occurred at 2p25-2q37, 4q21-q22, 4q28-q31.2, 4q31.3, 4q32-q35, 10p15-10q25, 13q11-q34, 16p13.3, 17p11.2, 19p13.2, 19p13.3

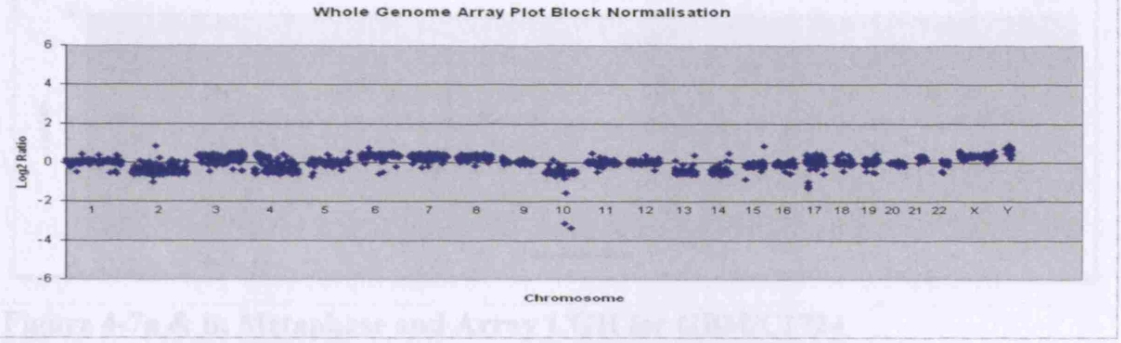
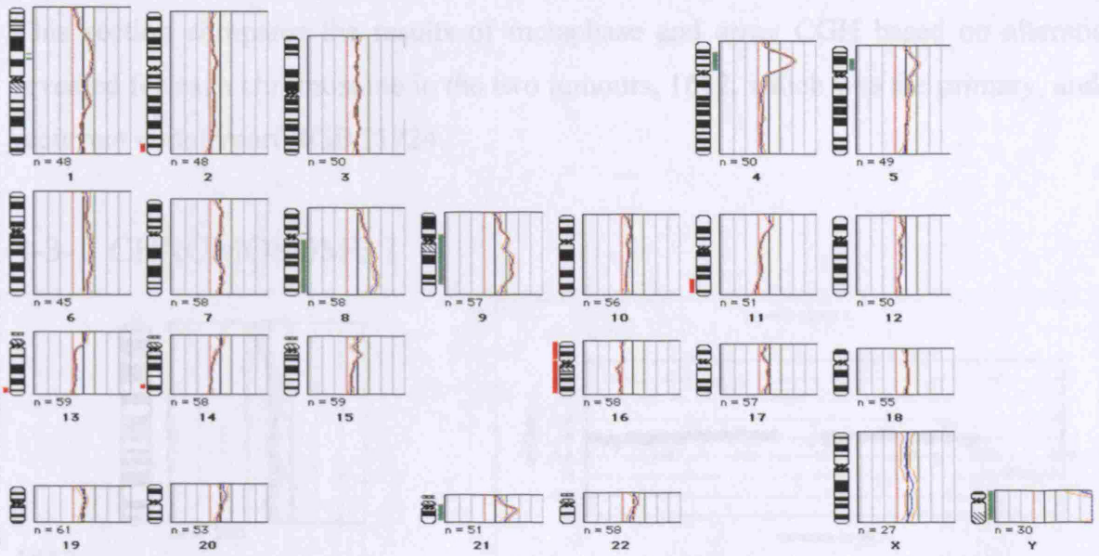


Figure 4-6a & b: Metaphase and Array CGH data for GBM/C1612

The whole genome array plot of PGBC1612 shows widespread copy number alterations in most of the chromosomes. Details of some prominent alterations reported by the software on CHRs 1612 and 1724 are discussed under individual chromosomes in the section that follows.

4-2-7 Tumour GBM/C1724



(7a): Gains were reported at 1p22, 4p11-p15.3, 5p12-p14, 8p11.2, 8q11.1-q24.3, 9p12-9q33, 21p11.1-21q22, Yp11.3-q12; and Losses at 2q35-q37, 11q22-q26, 13q32-q34, 14q24-q31, 16p11.1-p13.3, and 16p11.1-16q24

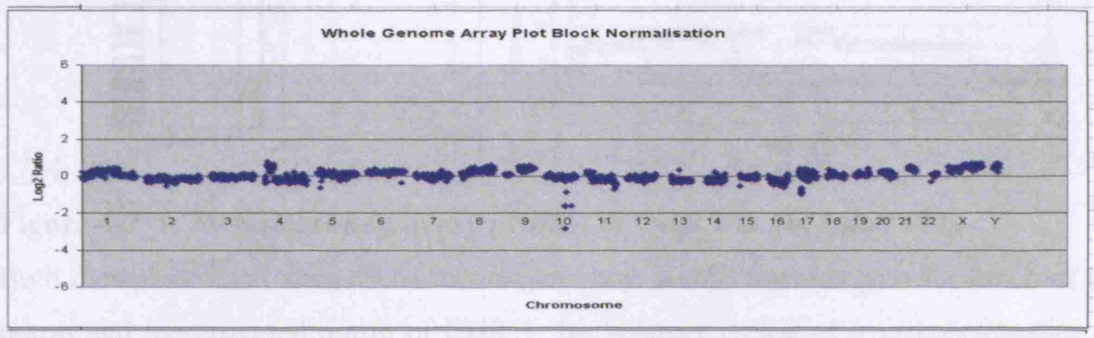


Figure 4-7a & b: Metaphase and Array CGH for GBM/C1724

The tumour RGBC1724, like its counterpart, 1612, also shows widespread copy number alterations in many chromosomes, but to a lesser degree. Metaphase CGH showed overrepresentation of CHR1, 4p, 5p, 6, 8, 9, and 21. On the other hand, several chromosomes were generally underrepresented, e.g., CHR 4q, 10q, 11q, 12q, 13q, 14q, 15q, and 16. These findings are validated by the array. Details of these alterations are presented with those of PGBC1612 in the following section.

4-3 Comparison of metaphase and array CGH data of GBMs/1612 and 1724

This section compares the results of metaphase and array CGH based on alterations revealed for each chromosome in the two tumours, 1612, which was the primary, and its recurrent counterpart RGBC1724.

4-3-1 CHROMOSOME 1

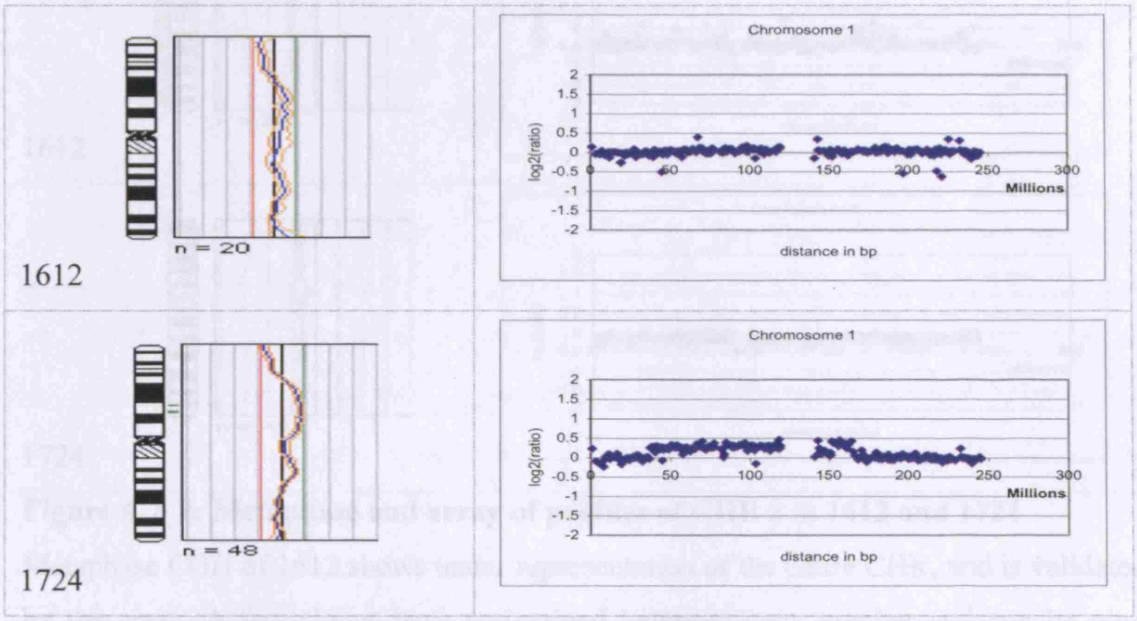


Figure 4-7_1: Metaphase and array profiles of CHR 1 in 1612 and 1724

Both metaphase- and array CGH techniques show a shift towards gain for much of the p-arm and the proximal q-arm of CHR 1, but a larger region of overrepresentation of copy number across the threshold is revealed by the arrays.

The array graph of PGBC1612 shows that most of the chromosome has balanced copy number except for several scattered clones that have either gained or lost copy number. Four prominently gained clones and a similar number that are lost on tumour 1612 are presented in Appendix 4.

CHR 1 of GBM/C1724 appears to have sustained three possible breakpoints, one on the p-arm whose boundaries are formed by clones RP4-731G4 (CHR1_36.32; fluorescence ratio (FR) 0.98) and RP11-204L3 (CHR1_39.81; FR 1.21). The two probable breakpoints in the q-arm are bordered by clones RP11-422P24 (CHR1_150.75; FR

1.05) and RP11-172I6 (CHR1_152.88; FR 1.38) - for the proximal breakpoint – and, the distal breakpoint, by clones RP11-541J2 (CHR1_160.96; FR 1.25) and RP4-702J19 (CHR1_164.73; FR 1.17).

4-3-2 CHROMOSOME 2

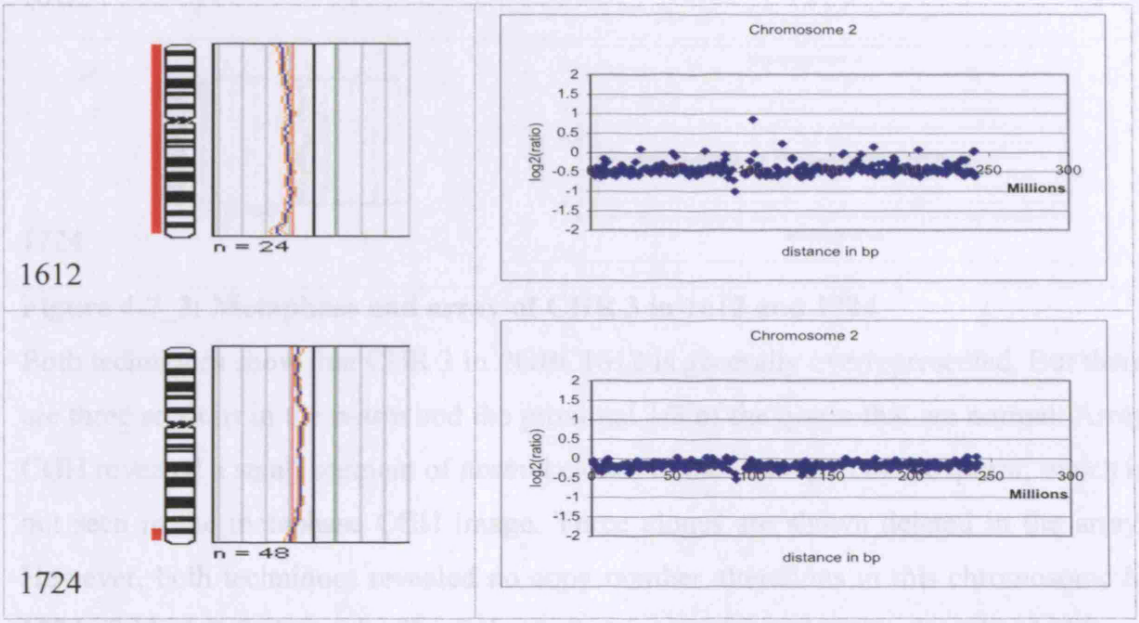


Figure 4-7_2: Metaphase and array of profiles of CHR 2 in 1612 and 1724

Metaphase CGH of 1612 shows under representation of the entire CHR, and is validated by the array. A few clones have maintained balanced copy number and can be seen straddling the ordinate position with the exception of clone RP11-299H21, which has apparently gained copy number. The entire CHR is similarly underrepresented, but to a lesser degree in tumour 1724 than in 1612. The details of eight clones that are prominently altered in both tumours are presented in Appendix 4.

4-3-3 CHROMOSOME 3

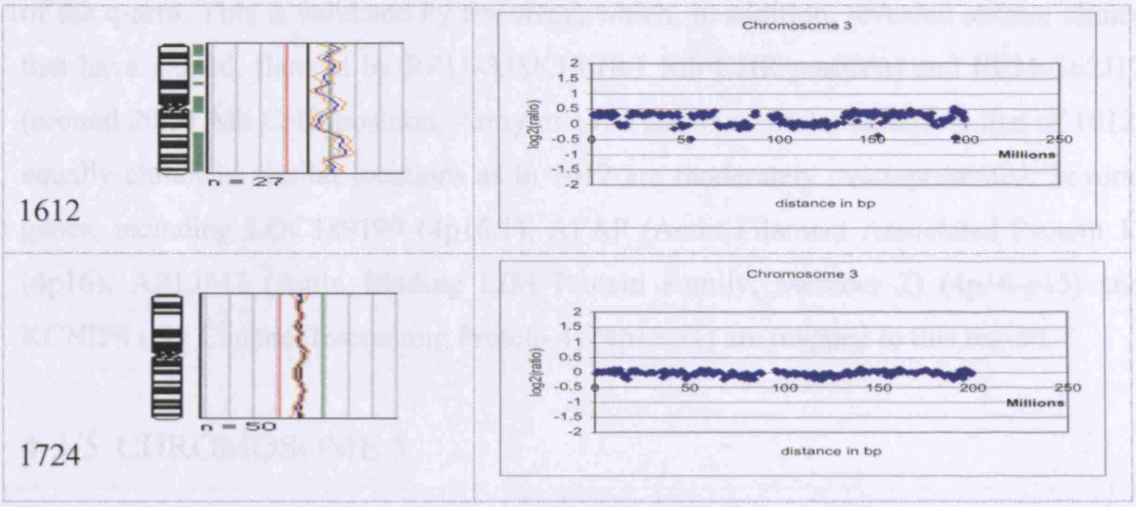


Figure 4-7_3: Metaphase and array of CHR 3 in 1612 and 1724

Both techniques show that CHR 3 in PGBC1612 is generally overrepresented. But there are three sections in the p-arm and the proximal 1/3 of the q-arm that are normal. Array CGH revealed a small segment of normal clones in the distal part of the q-arm, which is not seen in the metaphase CGH image. Three clones are shown deleted in the array. However, both techniques revealed no copy number alterations in this chromosome in 1724. Table 4-7_3 shows details of clones that are most prominently altered in 1612.

4-3-4 CHROMOSOME 4

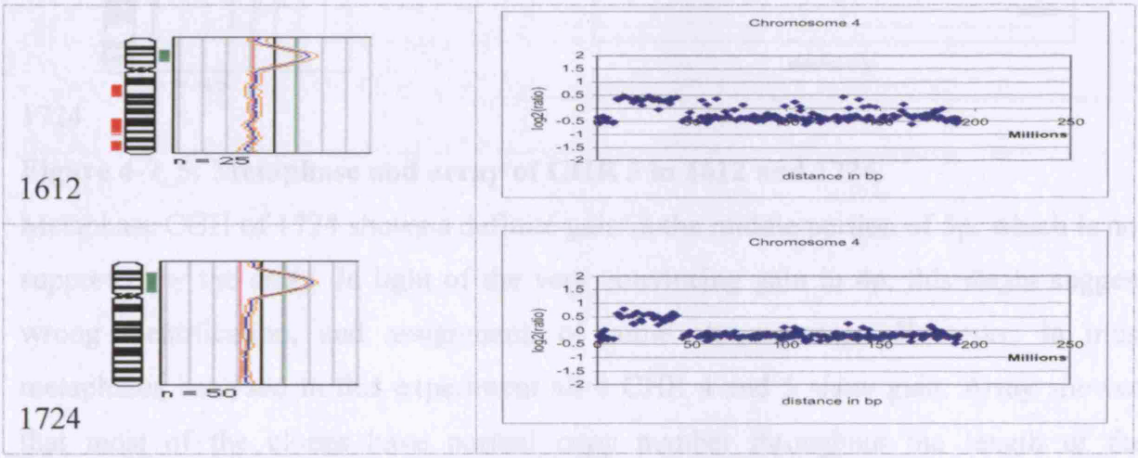


Figure 4-7_4: Metaphase and array of CHR 4 in 1612 and 1724

The metaphase CGH of both PGBC1612 and RGBC1724 show a region that has gained prominently for a distance of ~40Mb in the middle of the p-arm of CHR 4, which is validated in both cases by the array CGH. Both techniques also reveal loss of copy number throughout the q-arm. Although metaphase CGH shows three regions to be

deleted in 1612, the fluorescence ratio profiles are underrepresented for the entire length of the q-arm. This is validated by the array, which, in addition, revealed several clones that have gained, flanked by RP11-338K13 (8.1 Mb CHR position) and RP11-362J17 (around 20.51 Mb CHR position. Array of 1724 shows a similar pattern to that of 1612; equally clones in similar locations as in 1612 are moderately overrepresented. Several genes, including LOC389199 (4p16.1), AFAP (Actin Filament Associated Protein 1) (4p16), ABLIM2 (Actin Binding LIM Protein Family, Member 2) (4p16-p15) and KCNIP4 (Kv Channel Interacting Protein 4) (4p15.31) are mapped to this region.

4-3-5 CHROMOSOME 5

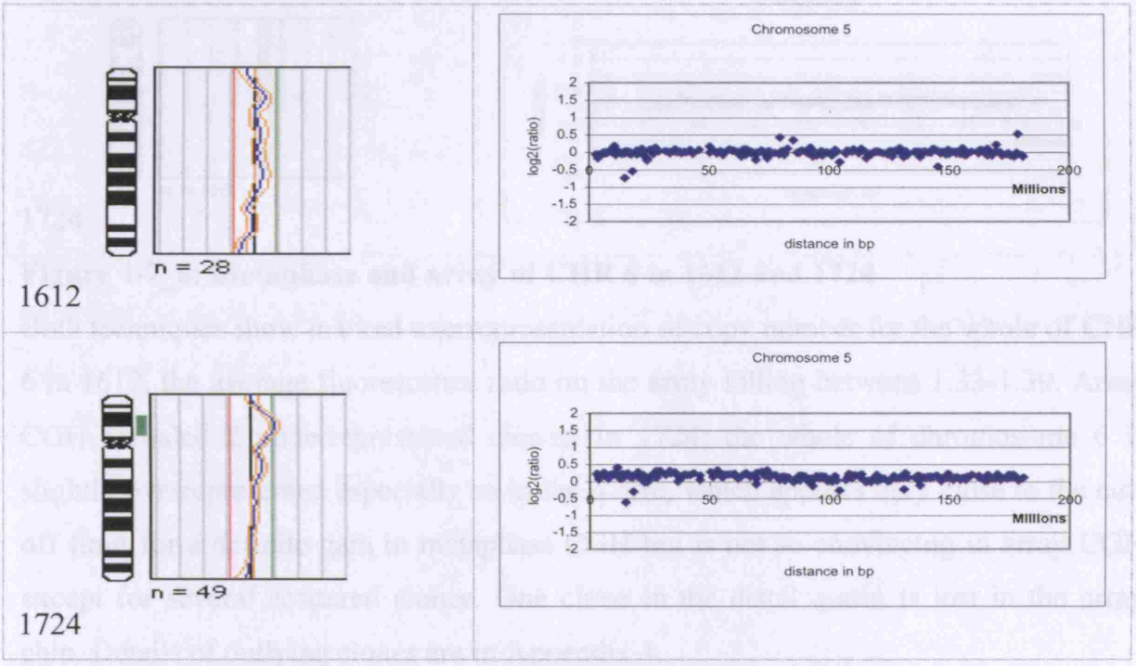


Figure 4-7_5: Metaphase and array of CHR 5 in 1612 and 1724

Metaphase CGH of 1724 shows a definite gain in the middle portion of 5p, which is not supported by the array. In light of the very convincing gain in 4p, this might suggest wrong identification, and assignment, of some chromosomes. However, in most metaphases analysed in this experiment all 4 CHR 4 and 5 show gain. Array showed that most of the clones have normal copy number throughout the length of the chromosome, both in 1612 and 1724. Two clones are shown prominently deleted in the p-arm of CHR 5 in both tumours while there are three prominently gained clones, 2 immediately proximal to the centromere in 1612, and the other more distal in the q-arm, around the 180Mb position. Several clones are moderately underrepresented in similar

locations in both tumours with no evidence shown in metaphase CGH. Details of some of the outlying clones are presented in Appending 4.

4-3-6 CHROMOSOME 6

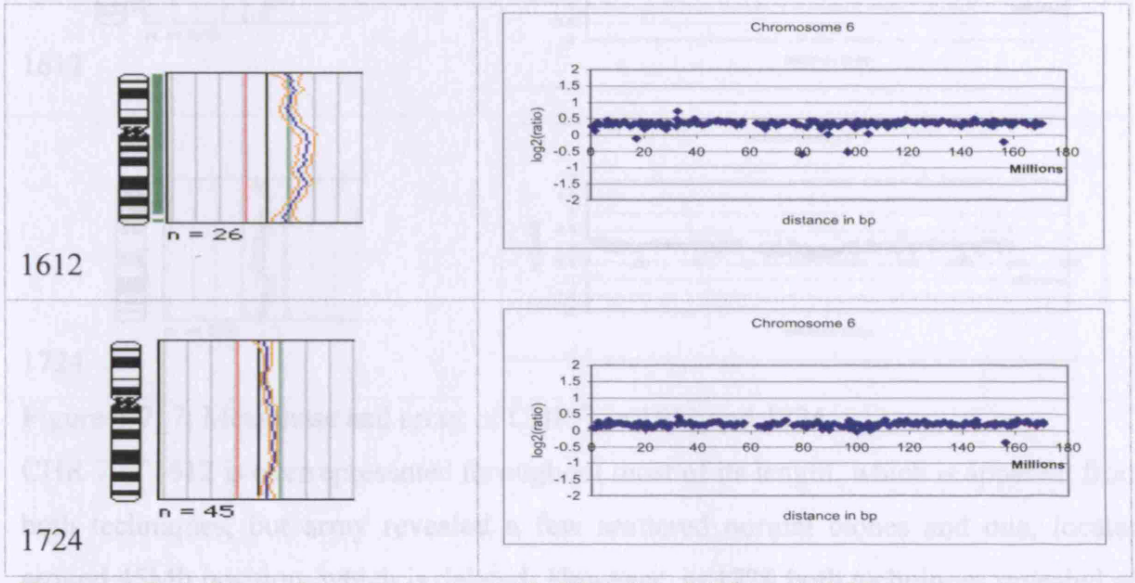


Figure 4-7_6: Metaphase and array of CHR 6 in 1612 and 1724

Both techniques show marked overrepresentation of copy number for the whole of CHR 6 in 1612, the average fluorescence ratio on the array falling between 1.33-1.39. Array CGH revealed 2 underrepresented clones. In 1724, the whole of chromosome 6 is slightly overrepresented especially so in the q-arm, which appears very close to the cut-off limit for a definite gain in metaphase CGH but is not so convincing in array CGH except for several scattered clones. One clone in the distal q-arm is lost in the array chip. Details of outlying clones are in Appendix 4.

4-3-7 CHROMOSOME 7

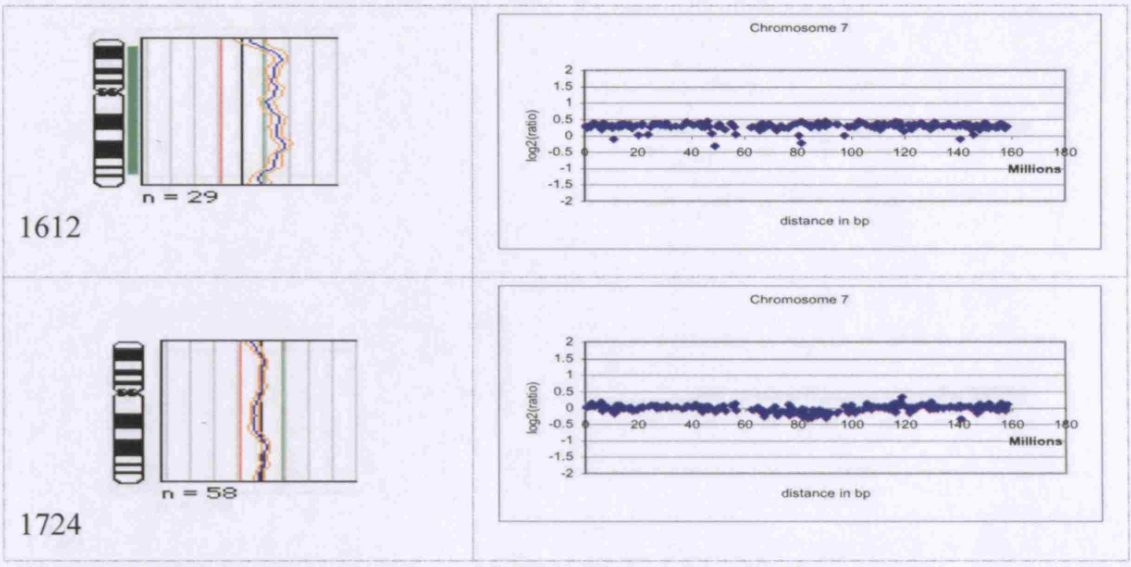


Figure 4-7_7: Metaphase and array of CHR 7 in 1612 and 1724

CHR 7 of 1612 is overrepresented throughout most of its length, which is apparent from both techniques, but array revealed a few scattered normal clones and one, located around 45Mb position, which is deleted. However, in 1724 both techniques revealed an underrepresented segment in the proximal q-arm that is roughly 40 MB. The same clone, located around 140 Mb, which is one of several that are underrepresented relative to the rest in chromosome 1612, is shown apparently deleted in 1724. Details of some of the most altered clones in CHR 7 are shown in Appendix 4.

4-3-8 CHROMOSOME 8

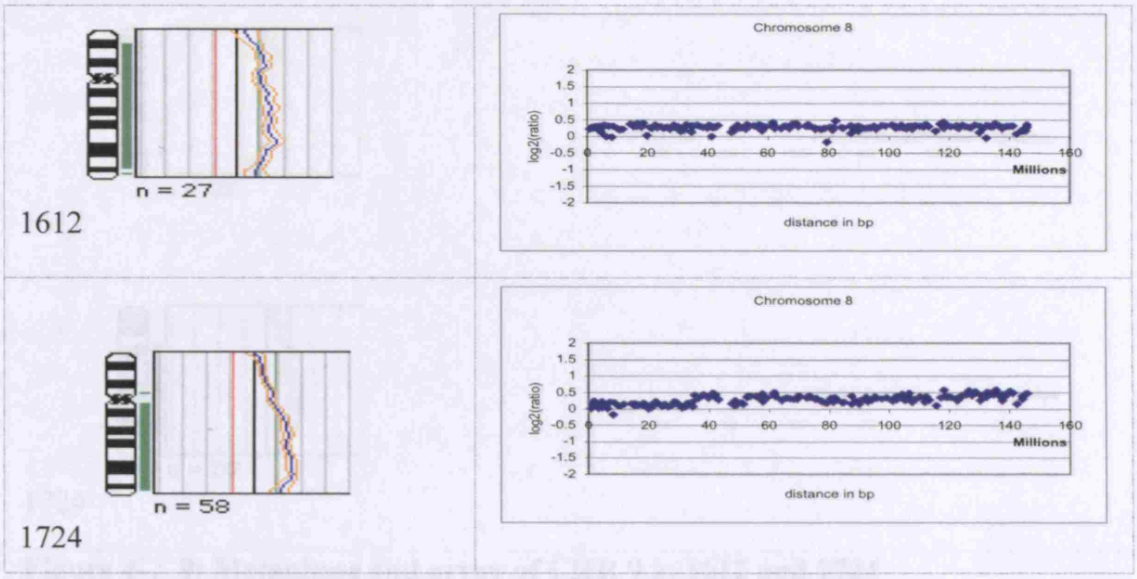


Figure 4-7_8: Metaphase and array of CHR 8 in 1612 and 1724

Both techniques are in agreement; in each case CHR 8 is shown overrepresented throughout almost the entire length in 1612 and the q-arm in 1724. There is a sharp rise in fluorescence around the 35.02 MB position, between clone RP1-144M5 (fluorescence ratio of 1.05) and RP1-155L11 (fluorescence ratio of 1.29), which is sustained in 22 contiguous clones for a distance of 7.1 MB. The demarcation between RP1-144M5 and RP1-155L11 may represent a possible translocation breakpoint in this CHR 8. This possibility is supported by results of MFISH analysis on RGBC1724, which found that CHR 8 was frequently involved in translocations. For example, der(8)t(8;22) was observed in 83% (5 out of 6) of the cells analysed, while der(8)t(8;12) occurred in 50% (3 out of 6) cells analysed. Other less frequent translocations involving CHR 8 were der(8)t(8;17) and der(8)t(8;19)t(19;21), each observed only in 1 out of 6 cells analysed (~17%).

4-3-9 CHROMOSOME 9

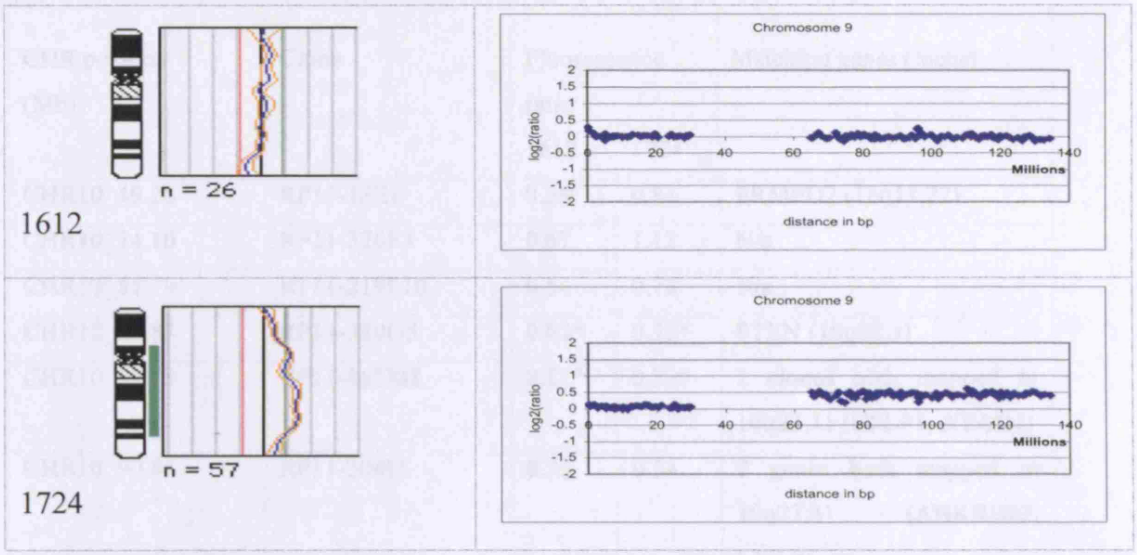


Figure 4-7_9: Metaphase and array of CHR 9 in 1612 and 1724

Both techniques agree in each case, showing normal CHR 9 in 1612 and overrepresented q-arm in 1724

4-3-10 CHROMOSOME 10

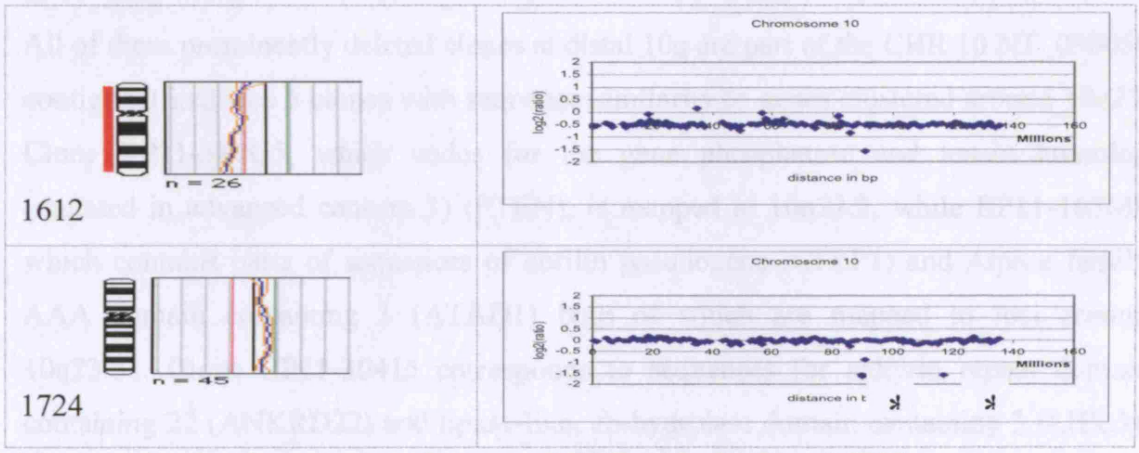


Figure 4-7_10: Metaphase and array of CHR 10 in 1612 and 1724

In 1612 both techniques show marked underrepresentation of the entire CHR 10, but with a couple of prominently deleted clones lying between 85-90 Mb. 1724 shows CHR 10 as generally normal except for several clones that are prominently deleted in the array chip. Three of these are located around 85-90 Mb, corresponding to the 10q23 region, and a fourth around 115-120 Mb region of the chromosome that corresponds to the coordinate 10q24-q25. Table 4-7_10 shows details of the most prominently deleted clones.

Table 4-7_10

CHR position (Mb)	Clone	Fluorescence ratio		Matching genes (locus)
		1612	1724	
CHR10_49.26	RP11-13E1	0.58	0.84	FRMPD2 (10q11.22)
CHR10_74.10	RP11-326F3	0.67	1.12	N/a
CHR10_85.79	RP11-219F10	0.54	0.78	N/a
CHR10_89.83	RP11-380G5	0.02*	0.32*	PTEN (10q23.3)
CHR10_89.75	RP11-165M8	0.11*	0.32*	2 clones both mapped to 10q23.31 (CFLP1, ATAD1)
CHR10_90.66	RP11-304I5	0.33	0.54	2 genes both mapped to 10q23.31 (ANKRD22, LIPL3)
CHR10_117.10	RP11-338L11	0.09*	0.33*	ATRNL1 (10q26)

*, The fluorescence ratio of these clones is so low that they do not appear on figure 4-7_10 but their positions can be seen on the whole-genome plot in figures 4-6b and 4-7b

All of these prominently deleted clones at distal 10q are part of the CHR 10 NT_030059 contig that includes 5 clones with sequence similarity to genes clustered around 10q23. Clone RP11-380G5, which codes for the gene phosphatase and tensin homolog (mutated in advanced cancers 1) (PTEN), is mapped to 10q23.3, while RP11-165M8 which contains parts of sequences of cofilin pseudogene (CFLP1) and Atpase family AAA domain containing 1 (ATAD1) both of which are mapped to loci around 10q23.31. Clone RP11-30415 corresponds to sequences for ankyrin repeat domain containing 22 (ANKRD22) and lipase-like, ab-hydrolase domain containing 3 (LIPL3), both also mapped at 10q23.31. The sixth' clone, RP11-338L11, contains sequences of the attractin-like 1 (ATRNL1) or melanocortin 4 receptor (MC4-R) that is thought to have critical roles in regulating glucose homeostasis (Haqq et al., 2003).

4-3-11 CHROMOSOME 11

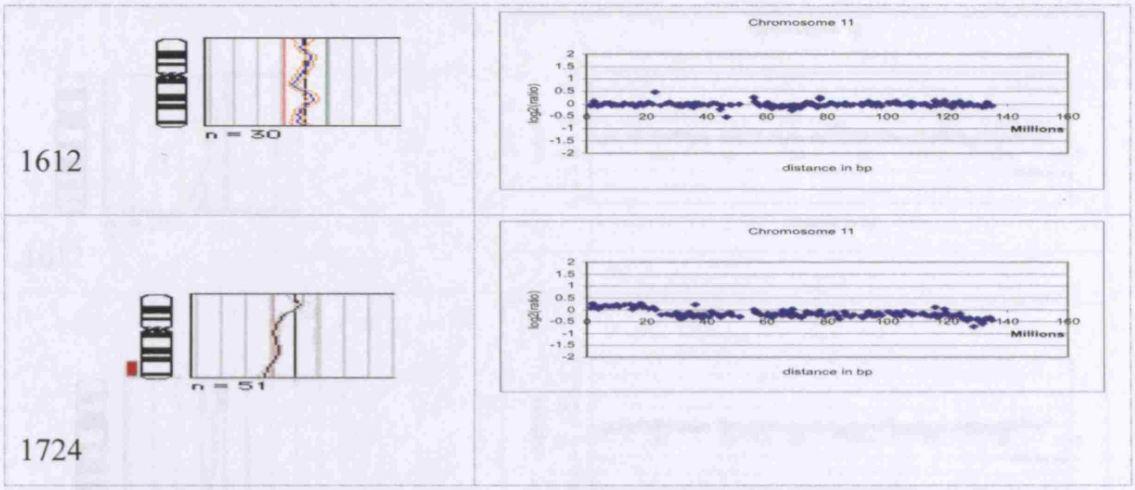


Figure 4-7_11: Metaphase and array of CHR 11 in 1612 and 1724

For tumour 1612, both techniques show mostly normal profiles, however array shows one prominently gained clone in the mid p-arm region and a deleted one in the heterochromatin region immediately above the centromere. In 1724 on the other hand, CHR 11 is generally underrepresented throughout most of its length except the upper half of 11p, which both techniques report as normal. The sharp break in continuity of the fluorescence ratio profiles seen in the middle of the p-arm could represent a translocation breakpoint. The probable translocation breakpoint is flanked proximally by clone RP11-34N19 (CHR11_22.56; mean fluorescence ratio of 1.02) and distally by RP11-72C9 (CHR11_24.54; mean fluorescence 0.89). The MFISH experiment of GBM/C1724 found der(9)t(9;11) translocations in 3 out of 6 (50%) cells analysed, which is a fairly a frequent occurrence that would warrant further study to establish genetic associations and their probable roles in the pathogenesis of this tumour. RP11-72C9 has sequences corresponding to those of the gene, leucine zipper protein 2 (LUZP2) that is mapped to 11p14.3. It is a chromosome segregation ATPase, thought to have roles in cell division and chromosome partitioning. As yet there are no known genes associated with sequences for clone RP11-34N19. There is a definite loss reported by both techniques around 11q24-q25. Table 4-7_11 contains details of some of the most altered clones

4-3-12 CHROMOSOME 12

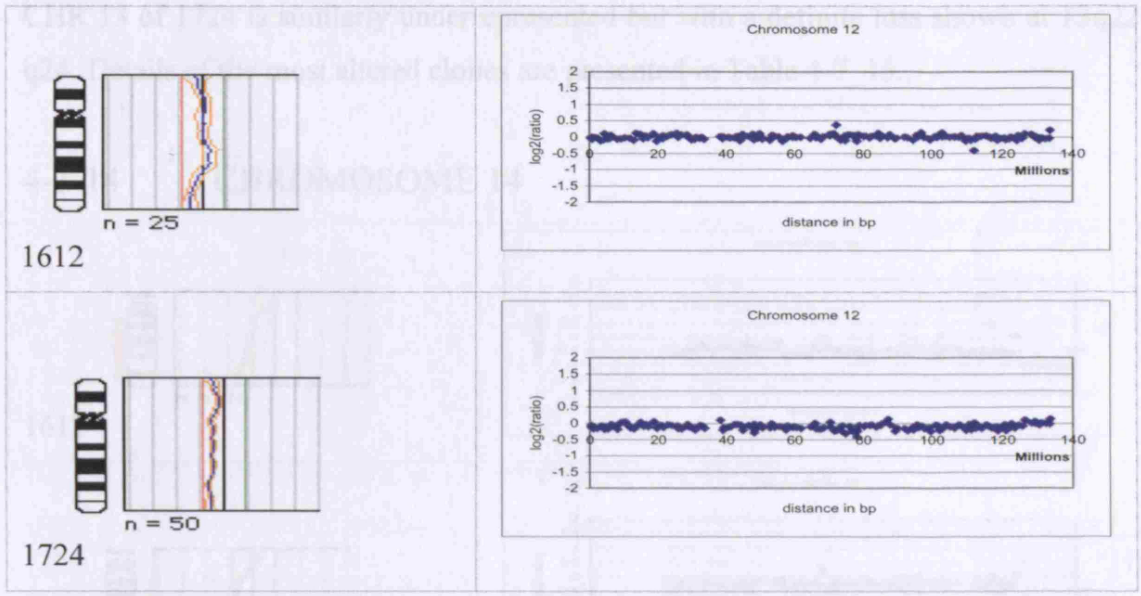


Figure 4-7_12: Metaphase and array of CHR 12 in 1612 and 1724

Metaphase and array CGH are in agreement in both tumours. The profiles of 1612 are shown as mostly normal, with two slightly overrepresented clones (one around 75 MB and one telomeric) and one deleted (at approximately 112 Mb position). Meanwhile, 1724 shows underrepresentation of the whole chromosome in both techniques. Several deleted clones are clustered around 95-100 Mb. Table 4-7_12, in Appendix 2, shows their details.

4-3-13 CHROMOSOME 13

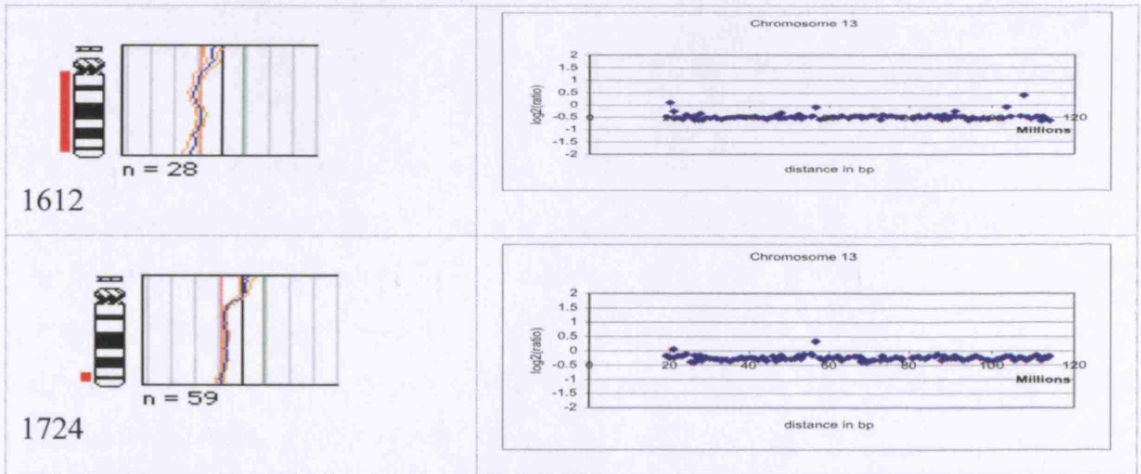


Figure 4-7_13: Metaphase and array of CHR 13 in 1612 and 1724

The whole of CHR 13 is shown deleted in metaphase CGH profile, which is supported by markedly underrepresented array profile of log2 T/N ratios. A few clones are shown

to have maintained balanced copy number while one at ~120 MB gained. The whole of CHR 13 of 1724 is similarly underrepresented but with a definite loss shown at 13q22-q24. Details of the most altered clones are presented in Table 4-7_13.

4-3-14 CHROMOSOME 14

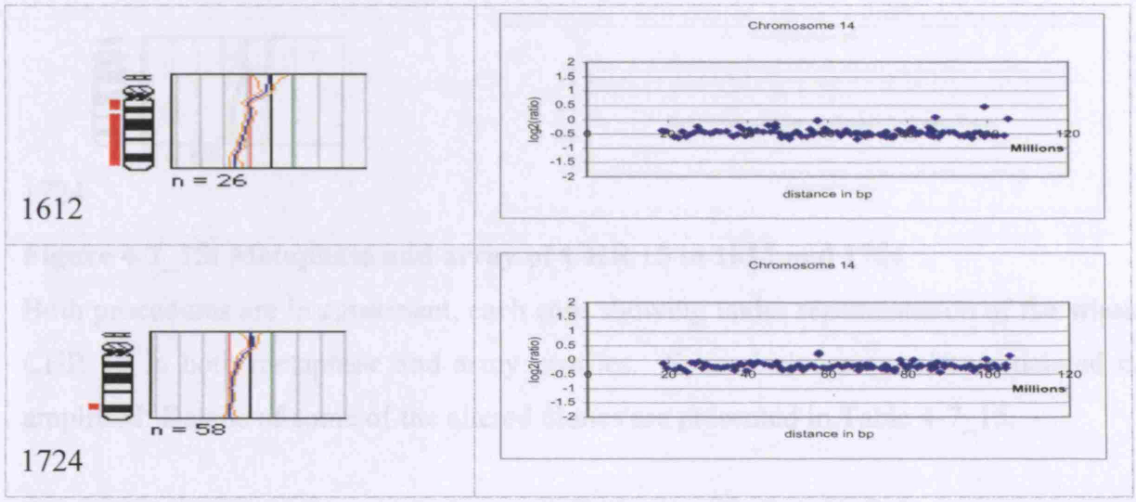


Figure 4-7_14: Metaphase and array of CHR 14 in 1612 and 1724

Like CHR 13, CHR 14 is also deleted across the entire length in 1612, which is confirmed by the array. A couple of clones are normal and one is shown to have gained. The whole chromosome 14 is similarly underrepresented in 1724 but only a short segment is reported deleted by the metaphase CGH. Some of the most altered clones are presented in Table -7_14.

4-3-15 CHROMOSOME 15

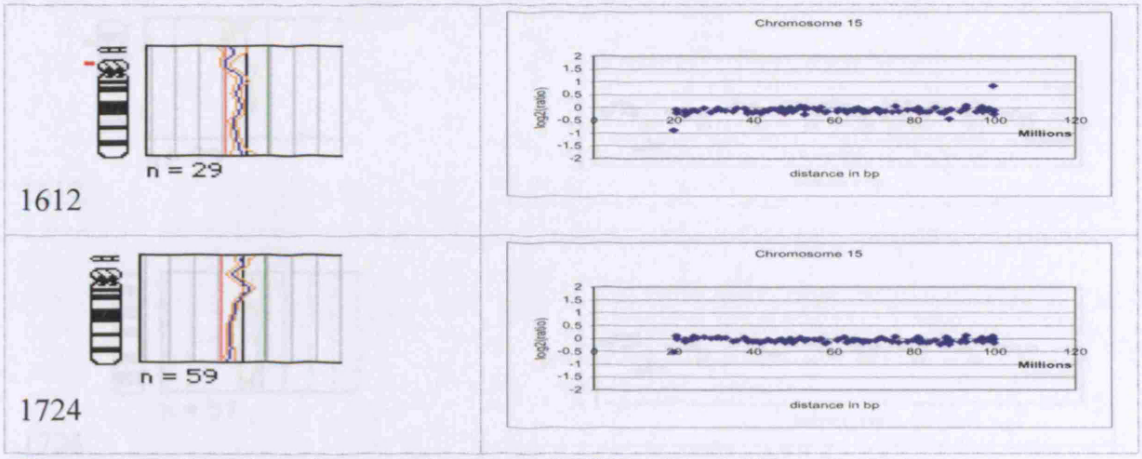


Figure 4-7_15: Metaphase and array of CHR 15 in 1612 and 1724

Both procedures are in agreement, each case showing under representation of the whole CHR 15 in both metaphase and array profiles. Several clones are shown deleted or amplified. Details of some of the altered clones are presented in Table 4-7_15.

4-3-16 CHROMOSOME 16

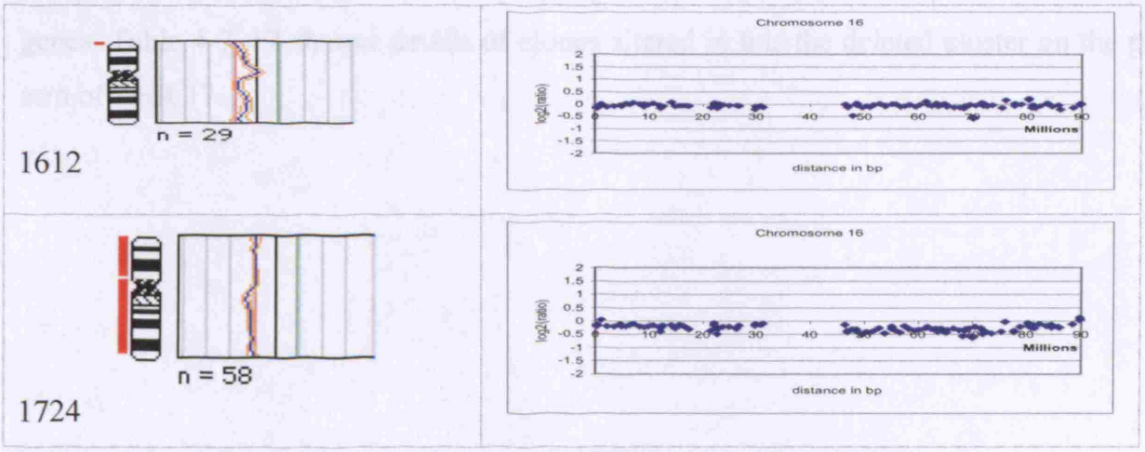


Figure 4-7_16: Metaphase and array of CHR 16 in 1612 and 1724

Both techniques show slight-to-moderate under representation of chromosome 16 in 1612, with a couple of clones shown deleted in the q-arm. However, the whole CHR 16 is shown deleted and prominently underrepresented, respectively, in metaphase and array profiles of 1724. A sample of the most altered clones in 1612 and corresponding fluorescence ratios of 1724 are shown in Table 4-7_16

4-3-17 CHROMOSOME 17

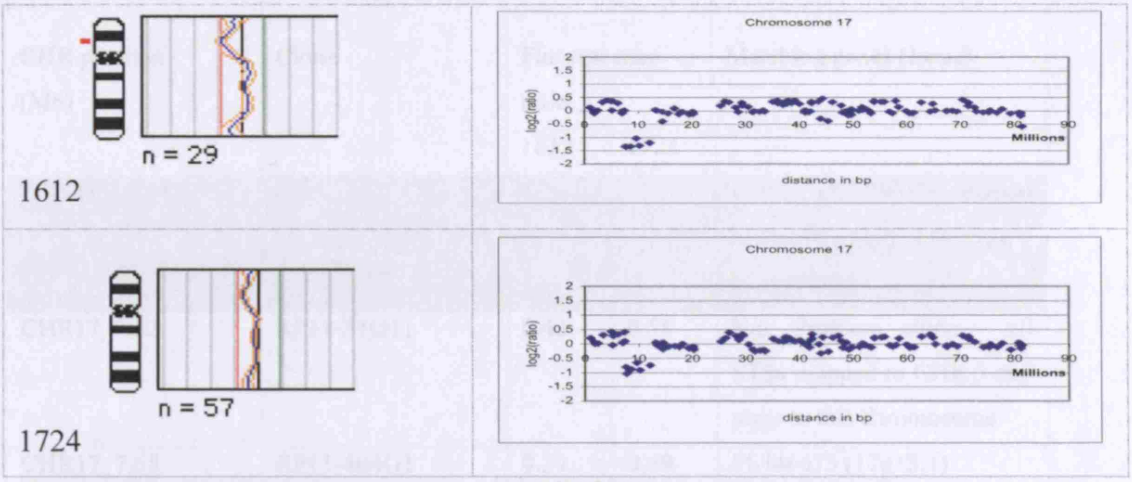


Figure 4-7_17: Metaphase and array of CHR 17 in 1612 and 1724

Both metaphase profiles show much of chromosome 17 to be within normal fluorescence cutoff range. However, the array shows that over 50 % of the clones in both tumours are overrepresented, with fluorescence ratios ranging between 1.25-1.36 on average. A few clones are underrepresented, the most prominent being around the 10 Mb position in both tumours, corresponding to the location of TP53 and several other genes. Table 4-7_17 shown details of clones altered in this the deleted cluster on the p-arm of CHR 17.

Table 4-7_17

CHR position (Mb)	Clone	Fluorescence ratio		Matching genes (locus)
		1612	1724	
*CHR17_7.518				Location of TP53 in relation to clones found in this array slide (plate)
CHR17_7.52	RP11-31O11	0.42	0.58	N/a; Problem clone – all STSs mapped to CHR 5 and none to this chromosome
CHR17_7.65	RP11-404G1	0.39	0.49	FLJ46675 (17p13.1)
CHR17_8.49	RP11-12H18	0.39	0.55	2 genes: FLJ32734 (17p13.1) and MYH10 (17p13)
CHR17_9.54	RP11-208F13	0.52	0.65	N/a
CHR17_10.15	RP11-401O9	0.39	0.61	2 genes: MYH8 (17p13.1) and MYH13 (17p13)
CHR17_12.02	RP11-471L13	0.44	0.60	MAP2K4 (17p11.2)

*The shaded row shows the CHR position of TP53 in relation to clones that are present in this array slide.

4-3-18 CHROMOSOME 18

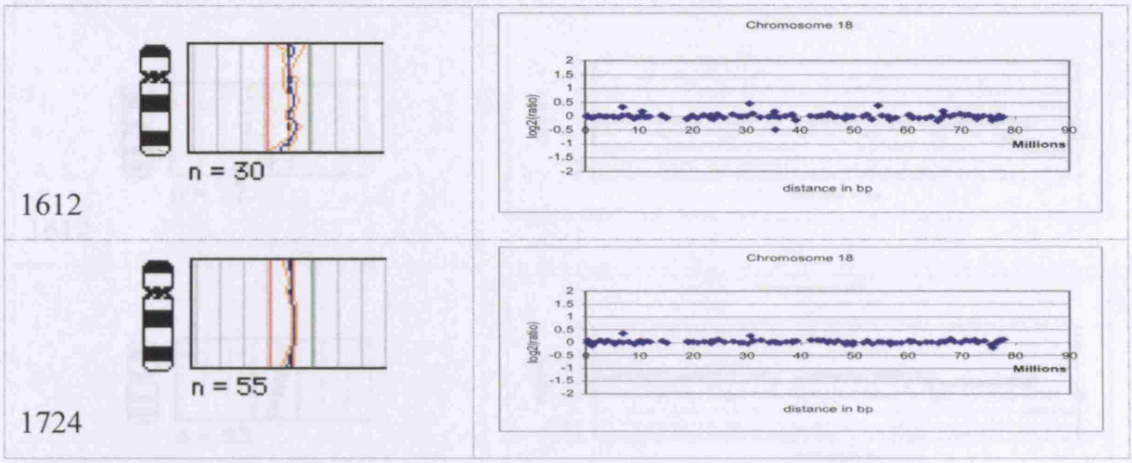


Figure 4-7_18: Metaphase and array of CHR 18 in 1612 and 1724

Metaphase and array experiments are in agreement, showing normal profiles in both 1612 and 1724. However, there are several outlying clones, most in similar positions in the two tumours. Details of these are shown in table 4, 7_18.

4-3-19 CHROMOSOME 19

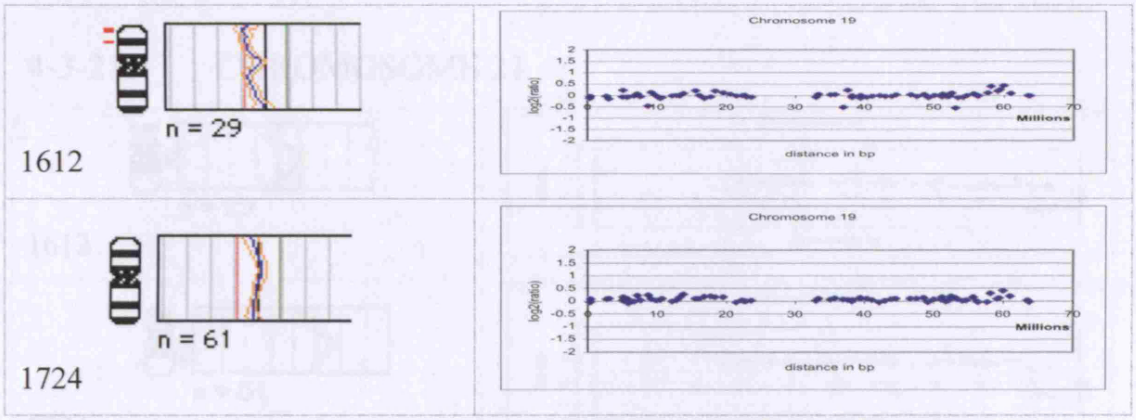


Figure 4-7_19: Metaphase and array of CHR 19 in 1612 and 1724

Metaphase CGH of 1612 showed deletion of 19p at 19p13.2 and 19p13.3 with an underrepresented fluorescence profile. The array log2 plot is slightly underrepresented in the distal ½ of the p-arm, while the remainder is normal except the distal ¼ of the q-arm where several clones are overrepresented. In addition, the array revealed 3 isolated clones that are deleted. The one in the p-arm appears to correspond to the deletion at 19p13.2; the other two deleted clones are located around 37 Mb and 53 Mb respectively. In 1724, both techniques showed normal fluorescence ratio profiles, except that the array revealed overrepresentation of the same set of clones seen in 1612. Details of these prominently altered clones are presented in Table 4, 7_19

4-3-20 CHROMOSOME 20

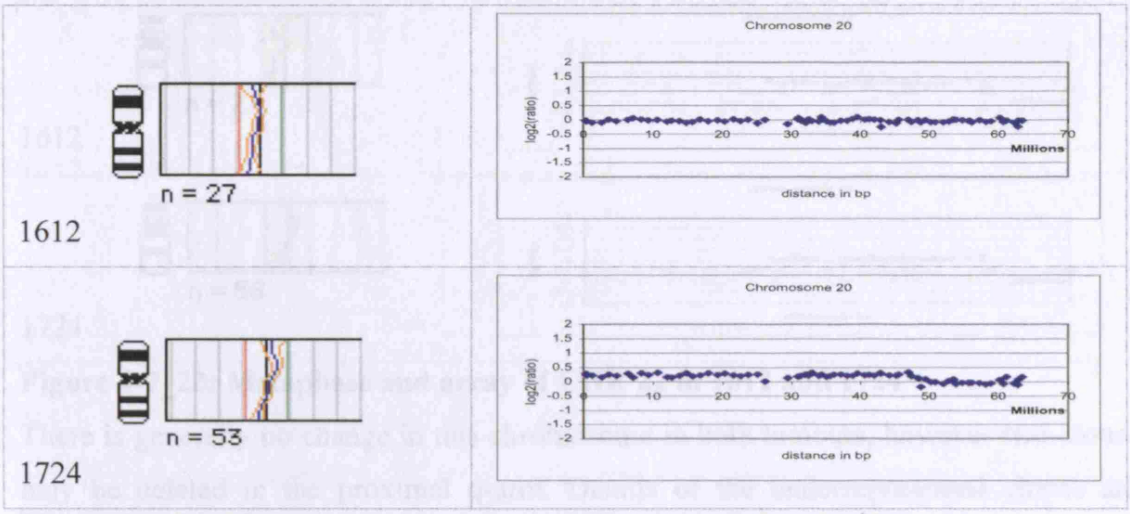


Figure 4-7_20: Metaphase and array of CHR 20 in 1612 and 1724

Both techniques show normal CHR 20 profiles in 1612, but with the array showing a couple of underrepresented clones in the distal q-arm. However, both metaphase and array plots reveal overrepresentation of the p-arm and proximal ½ of the q-arm; the distal ½ is normal except for a couple of overrepresented clones at the very end.

4-3-21 CHROMOSOME 21

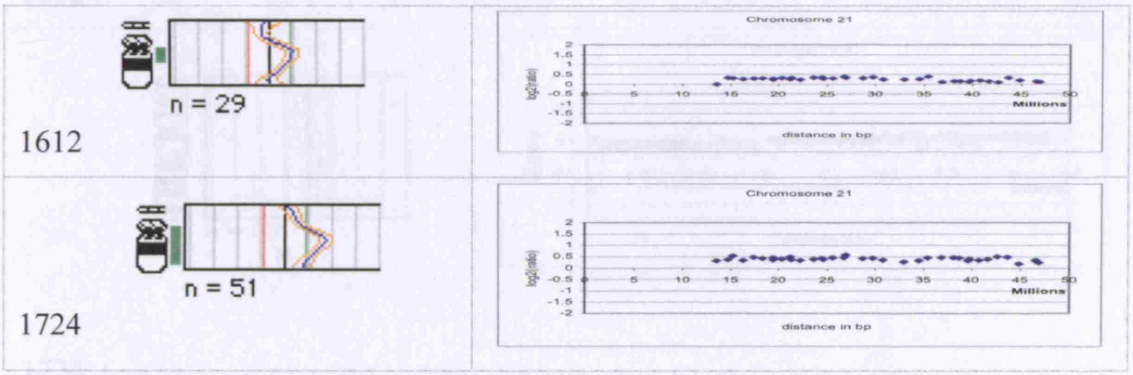


Figure 4-7_21: Metaphase and array of CHR 21 in 1612 and 1724

CHR 21, both in 1612 and 1724, were generally overrepresented, with gains in the q-arm.

4-3-22 CHROMOSOME 22

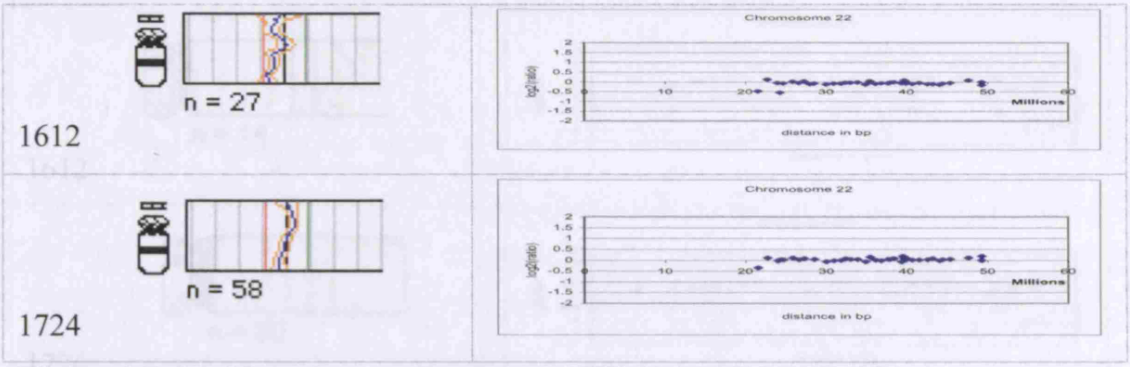


Figure 4-7_22: Metaphase and array of CHR 22 in 1612 and 1724

There is generally no change in this chromosome in both tumours, however two clones may be deleted in the proximal q-arm. Details of the underrepresented clones are presented in Table 4, 7_22.

4-3-23 X-CHROMOSOME

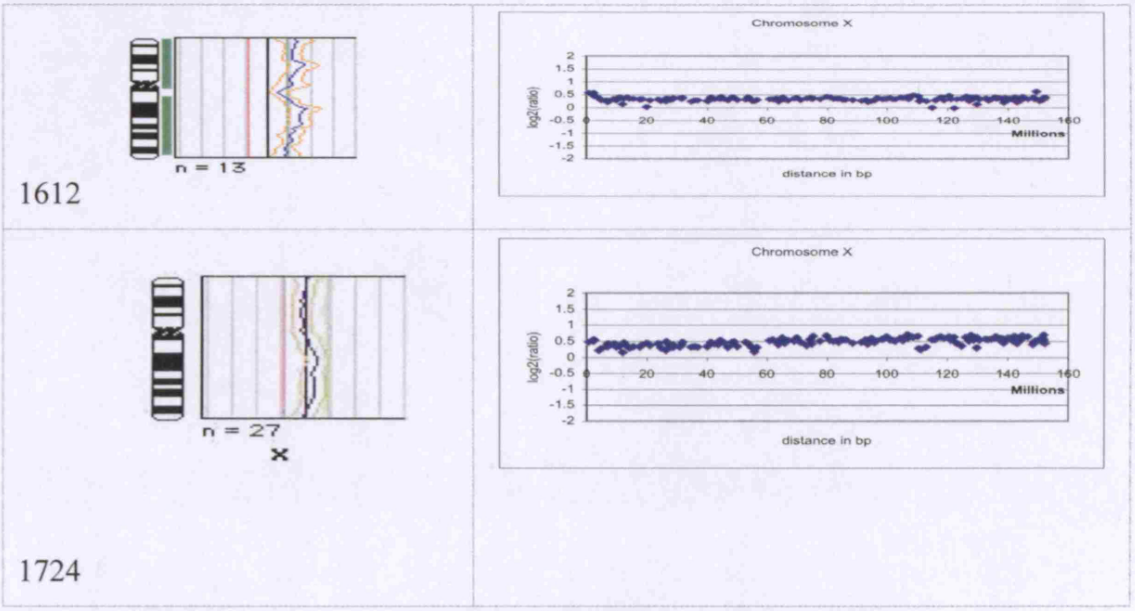


Figure4-7_23: Metaphase and array of CHR-X in 1612 and 1724

The entire X-chromosome is gained in the metaphase and is overrepresented throughout in the array. In 1724, the metaphase only shows overrepresentation in the long-arm, while the chip shows the entire chromosome to be overrepresented and is clearly gained.

4-3-24 Y-CHROMOSOME

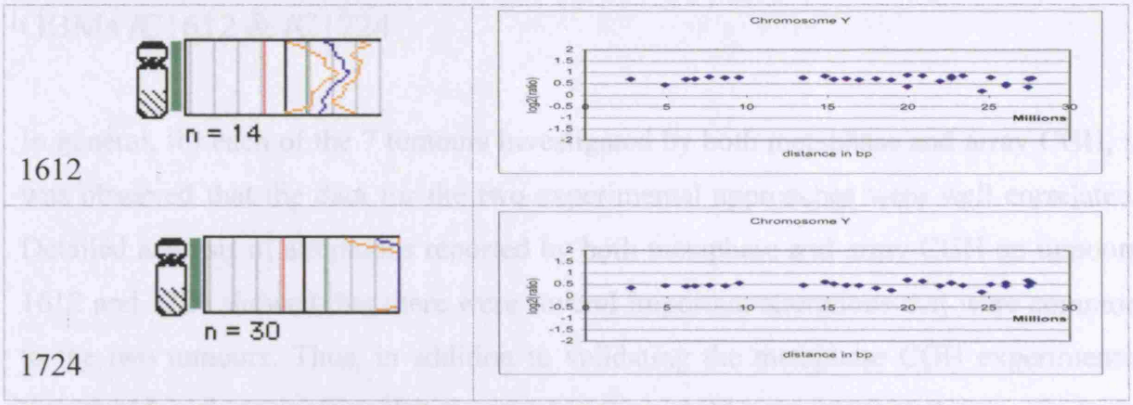


Figure 4-7_24: Metaphase and array of CHR-Y in 1612 and 1724

In both tumours, metaphase and array techniques show this chromosome to be generally overrepresented and gained entirely.

4-4 Summary on comparisons data of metaphase and array data for GBMs /C1612 & /C1724

In general, for each of the 7 tumours investigated by both metaphase and array CGH, it was observed that the data for the two experimental approaches were well correlated. Detailed analysis of alterations reported by both metaphase and array CGH on tumours 1612 and 1724 showed that there were several important alterations that were common to the two tumours. Thus, in addition to validating the metaphase CGH experiments, this would in deed confirm that the two tumours could have originated from the same individual. Metaphase CGH of 1612 and 1724 revealed a large number of alterations in both tumours. Table 4_8 is a diagrammatical representation of alterations in both tumours.

4-4-1 Table 4_8: Comparison of CNAs revealed by metaphase CGH in GBMs/1612 & /1724

	1612_Gain	1612_Loss	1724_Gain	1724_Loss
CHR1p			1p22	
CHR1q				
CHR2p		2p26-q37		
CHR2q		2p26-q37		2q35-q37
CHR3p	3p12-q1.2, 3p14, 3p24-p26			
CHR3q	3q21-q28			
CHR4p	4p13-p15.2		4p11-p15.3	
CHR4q		4q21-q22, 4q28-q31.2, 4q31.3, 4q32-q35		
CHR5p			5p12-p14	
CHR5q				
CHR6p	6p25-q25			
CHR6q	6p25-q25, 6q27			
CHR7p	7p22-q35			
CHR7q	7p22-q35,			
CHR8p	8p22-q24.2		8p11.2-q24.3	

CHR8q	8p22-q24.2, 8q24.3		8p11.2-q24.3	
CHR9p			9p12-q33	
CHR9q			9p12-q33	
CHR10p		10p15-q26		
CHR10q		10p15-q26		
CHR11p				
CHR11q				11q22-q25
CHR12p				
CHR12q				
CHR13q		13q11-q34		13q32-q34
CHR14q		14q11.1-q12, 14q13-q32		14q24-q31
CHR15q				
CHR16p		16p13.3		16p11.2-p13.3
CHR16q				16q11.2-24
CHR17p		17p11.2		
CHR17q				
CHR18p				
CHR19p		19p13.2, 19p13.3		
CHR19q				
CHR20p				
CHR20q				
CHR21q	21q11.2-q21		21q11.1-q22	
CHR22q				
CHR-Xp	Xp22.3-q12			
CHR-Xq	Xp22.3-q12, Xq13-q28			
CHR-Yp			Yp11.3-q34	
CHR-Yq			Yp11.3-q34	

Table 4_8: Metaphase CGH data on alterations in 1612 and 1724. Shaded rows show regions of common alterations in the 2 tumours. Green represents gain of DNA copy number while orange /brown represents regions that were shown as deleted.

4-5 Array CGH data showing DNA copy number alterations at 19 loci of some of the genes associated with development of astrocytomas - both hereditary and sporadic.

As mentioned in the introductory chapter, ~5% of astrocytomas arise in association with hereditary syndromes (e.g., neurofibromatosis or tuberous sclerosis), as a result of germline mutations in the TSGs that cause the syndromes. However, the majority of astrocytomas appear to arise spontaneously, following somatic mutations of other cancer predisposing genes. Thus, some cancer-associated genes are believed to cause only hereditary astrocytomas, while others appear to have roles in both hereditary and spontaneous astrocytomas. At least 19 loci harbour genes implicated in development of astrocytomas in general. Table 4_9, shows the fluorescence ratios of clones (from array-CGH) that are mapped within these loci, or in their vicinity, in the 7 tumours further investigated by this method.

Table 4_9: ArrayCGH data on clones matched to genes that are implicated in astrocytoma development

Gene/Locus	Clones & Nucleotide coordinates	Fluorescence rations (Mean) C1752	C1706	C1510	C160	S2093	C1612	C1724
PDGFA: 7p22 NC.503423-526007	CTB-164D18 7_254609.5	1.02	1.02	1.14	1.08	1.23	1.27	1.03
PMS2: 7p22 NC.5979396-6015263	RP11-449P15 7_727016.5	0.98	1.05	1.03	1.07	1.08	1.21	1.06
EGFR: 7p12 NC.7518224-7531642	RP4-733B9 7_6978224.5	1.05	1.09	1.16	1.06	1.27	1.35	1.07
	RP11-505D17 7_7780751	1.00	1.09	1.19	1.03	1.18	1.34	1.02
CDKN2A/2B: 9p21 NC.5979396-6015263	RP11-509D8 9_501649	1.00	1.07	1.09	1.08	0.83	1.07	1.07
	RP11-218I7 7_6070088.5	1.04	1.04	1.03	1.03	0.82	1.03	1.00
PTEN: 10q23.3 NC.89613175-89718512	RP11-165M8 10_89754667	0.91	0.94	1.00	0.96	0.85	0.11	0.33
	RP11-380G5 10_89838961.5	1.03	1.02	1.00	0.97	0.91	0.6	0.32
	RP11-765C10 10_89938145.5	1.00	1.01	1.03	0.96	0.86	0.02	0.14
	RP11-304I5 10_90666405.5	-	0.94	0.99	1.02	0.90	0.33	0.55
DMBT1: 10q26.13 NC.124310171-124393242	RP11-436O19 10_124317277	1.34	1.00	0.93	1.13	0.84	0.6	0.92
CDK4: 12q14.3	RP11-183H16 12_56354120.5	0.98	0.99	0.98	0.94	0.85	0.9	0.96

NC.56428270-56432431								
Gene/Locus	**Clones & Nucleotide coordinates**	**C1752**	**C1706**	**C1510**	**C160**	**S2093**	**C1612**	**C1724**
MDM2 (PAP1B): 12q15	RP11-328H16 12_67031985.5	0.99	1.03	1.01	1.05	1.06	1.04	0.86
NC.67489255-67520481								
	RP11-542B15 12_67594405	0.98	0.99	0.99	0.96	1.03	0.99	0.91
RB1: 13q14.2	RP11-305D15 13_47867886.5	1.14	1.05	1.06	1.13	1.10	0.75	0.89
NC.47775884-47954027								
	RP11-174I10 13_47917948.5	0.97	1.06	1.07	0.90	0.98	0.70	0.88
TP53: 17p13.1	RP11-199F11 17_7520901.5	0.94	0.92	0.97	0.86	0.74	0.99	0.58
NC.7518224-7531642								
	RP11-404G1 17_7650784	0.93	0.94	0.92	0.94	0.82	0.40	0.50
TP73: 1p36.32	RP4-785P20 1_2868729.5	1.14	0.96	0.94	0.98	1.01	0.92	0.93
NC.3597096-3639716								
	RP1-37J18 1_4103538	1.06	0.96	0.94	0.96	0.98	0.94	0.90
19q								
MSH2: 2p21-22	RP11-436K12 2_47690350.5	1.02	0.96	0.95	0.99	0.93	0.71	0.91
NC.47483767-47760014								

	RP5-960D23 2_47939947	0.96	0.97	0.94	0.99	0.77	0.69	0.90
Gene/Locus	**Clones & Nucleotide coordinates**	**C1752**	**C1706**	**C1510**	**C160**	**S2093**	**C1612**	**C1724**
PMS1: 2q31-q33	CTC-444N24 2_190075192	1.26	1.31	1.21	1.44	1.23	0.90	0.96
NC.190426900-190437086								
	RP11-455J20 2_190621121	1.02	1.09	1.07	0.99	0.99	0.79	0.97
MLH1: 3p21.3	RP11-491D6 3_36887415	1.06	1.03	1.01	1.00	0.96	1.27	1.04
NC.37020896-37028594								
	RP11-331G2 3_39061581.5	0.99	0.99	0.96	1.03	0.99	1.27	1.04
APC: 5q21-22	RP11-3B10 5_112080205.5	0.97	1.04	0.98	1.04	1.02	1.00	1.12
NC.112101483-112209835								
	CTC-1554D6 5_112161361	1.04	1.09	1.05	1.17	1.07	0.99	1.28
PTCH: 9q22.3	RP11-80H12 9_97251057	1.01	0.96	1.00	0.93	1.08	1.06	1.31
NC.97283932-97319160								
	RP11-75J9 9_97978853	1.01	0.97	1.00	0.92	1.06	1.00	1.36
TSC1: 9q34	GS1-135I17 9_134247737	0.96	0.97	0.98	0.99	0.88	1.01	1.37
NC.134771939-134809841								
MLH3: 14q24	RP11-368K8 14_74223867.5	0.99	0.99	0.96	1.07	0.99	0.71	0.78
NC.74550220-74587988								
	RP11-361H10 14_74663588.5	0.99	0.96	0.98	1.01	0.94	0.69	0.81
TSC2: 16p13.3	RP11-161M6 16_1008467.5	0.99	0.92	0.95	0.91	0.95	1.00	1.05
NC.2037991-2078714								

	RP11-304L19 16_2167457	0.94	0.94	0.92	0.93	0.83	0.92	0.81
Gene/Locus	**Clones & Nucleotide coordinates**	**C1752**	**C1706**	**C1510**	**C160**	**S2093**	**C1612**	**C1724**
NF1: 17q11.2	RP11-138P22 17_26048765	0.99	0.94	0.99	0.91	0.89	1.25	1.12
NC.26470278-26670503								
	RP11-192H23 17_26744090.5	0.94	0.93	0.94	0.95	0.84	1.28	1.33
	RP11-229K15 17_29200556	0.95	0.95	0.98	0.94	0.78	1.27	1.36
	RP11-104I20 17_27994182	0.94	1.02	0.94	0.76	0.92	1.13	1.23
NF2: 22q12.2	RP1-329J7 22_205047	1.03	1.04	0.95	1.13	0.98	1.01	1.02
NC.28329565-28424585								
	CTA-57G9 17_27894024.5	0.94	0.91	0.95	0.93	0.8	0.96	1.03

Caption for table 4_9:

Light-green shading represents gains, beige shading represents loss, and Red font shows borderline fluorescence ratios - on the lower side of normal.

5 CHAPTER 5: Results of MFISH Analysis of GBM/C1724

Multicolour fluorescent-*in situ* hybridisation (MFISH) was performed on chromosome spreads made from RGBC1724, which was a high malignancy grade astrocytoma (WHO Grade 4). The M-FISH was kindly carried out and analysed in Dr Denise Sheer's lab by Radost Vatcheva, primarily because the software for manipulating the images was not available at the Galton lab. However, I was present at the experiments and participated in the analysis.

The point of doing this experiment was to see how the results related to the CGH experiments, and also to determine the number of chromosomes in each cell and see if any translocations of potential interest could be observed. It was also hoped to determine whether the gain on 4p was present on double minutes (DMs) or an obvious homogeneous staining region (HSR), which are believed to signify gene amplification (Bigner et al., 1987; Kleinschmidt-DeMasters et al., 2004; Arjona et al., 2005).

5-1 MFISH findings

The data presented here include one metaphase spread (fig 5:1 (a)), one karyotype from a different cell (fig. 5:1 (b)) and a summary table of the overall findings (Table 5:1). The major finding was the very large number of chromosomes in each cell and the heterogeneity between the six cells examined. The number of chromosomes could certainly explain the general slight over- or under-representation of particular chromosomes noted by CGH, but the heterogeneity makes comparison of copy number impossible. It was frustrating that no explanation for the clear gain of 4p material previously seen could be found. Simple chromosomal alterations in the form of deletions were noted to occur less frequently in chromosomes 1 (1p), 3, 4, 6, 7, 8, 9, 10, 11, 12 (12p), 16, 18 and the X-chromosome, while simple gains, in comparison to losses, were much less commonly encountered. For example simple gains were seen in chromosome 10, 16 and 20. More common, however, and potentially of great interest, was the occurrence of simple rearrangements (involving two chromosomes) and some more complex rearrangements (involving three or more chromosomes). Of particular

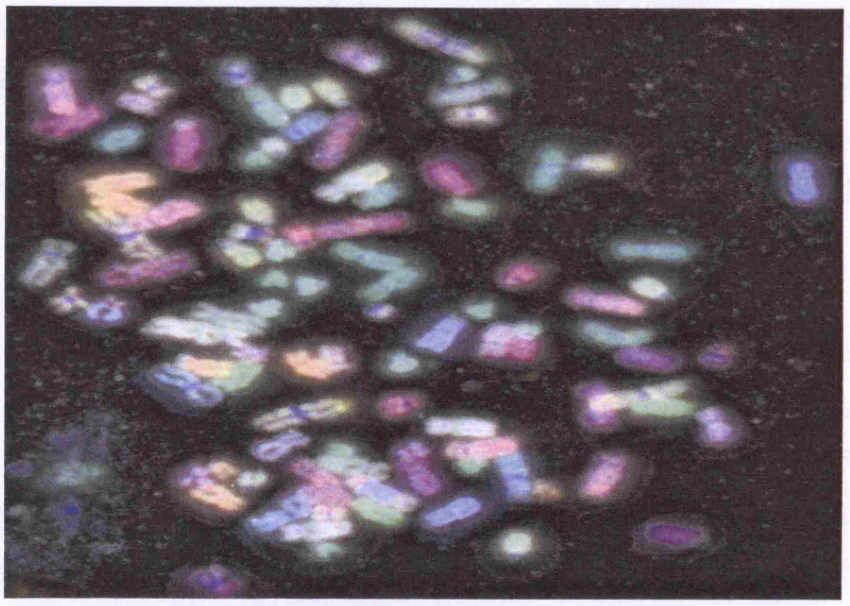
note were two translocations seen in several cells. One of them was a translocation between chromosomes 8 and 22, where the former was the recipient in der(8)t(8;22) which was observed in 5 out of 6 (83%) cells analysed. The other was der(X)t(16;X)t(17;X), which was observed in 4 cells (67%). Thus, in decreasing frequency rearrangements found in two or more of the six cells analysed were: der(8)t(8.22) (83%), der(X)t(16;X)t(17;X) (67%), der(8)t(8;12) (50%), der(9)t(9;11) (50%), der(1)t(1;8), (33%), der(5)t(5;22) (33%), der(6)t(6;13) (33%), der(7)t(7;10) (33%), der(7)t(7;X) (33%), der(9)t(6;9) (33%), der(10)t(10;15) (33%), der(20)t(7;20) (33%). These and other rearrangements/alterations observed only in six cells of the same tumour are shown in Table 5-1 (page 181).

Explanation for table 5:1

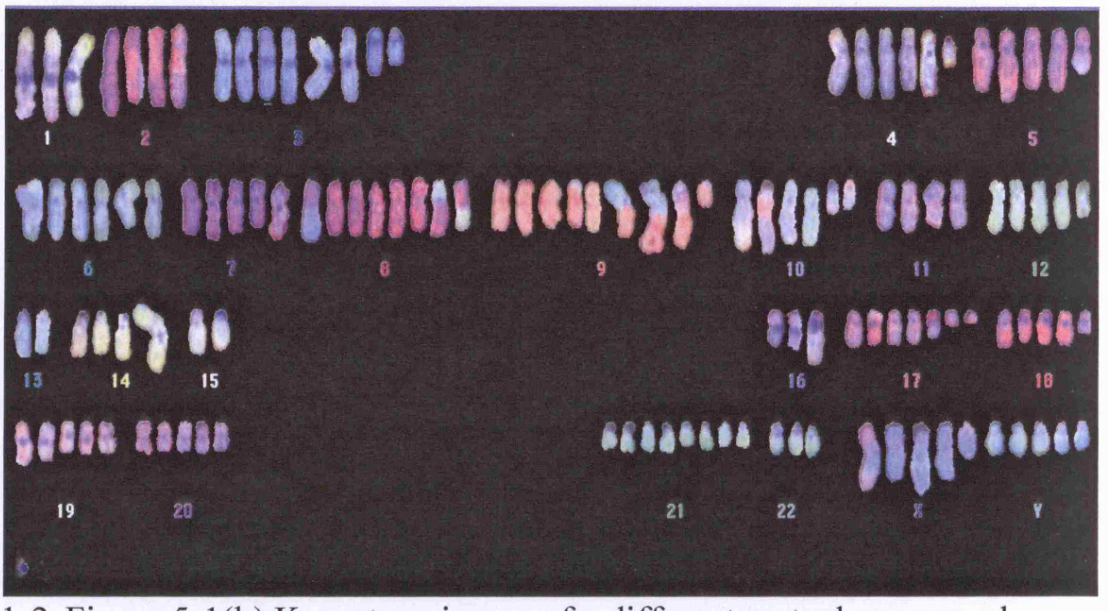
The column of frequency indicates the number of times an aberration was observed in the six cells analysed. Where no figure is given, the aberration was observed just once. T & t: translocation; Del, deletion; Der, derived/(indicating the recipient chromosome of an additional copy or chromosome segment).

5-1-1 Table 5-1: List of cytogenetic aberrations revealed by MFISH on GBM/C1724

T(3;16)t(3;5)		*Der(9)t(6;9)*	*2*
T(7;10;3)		Der(9)t(9;18)	
T(3;8)		*Del(9)*	*5*
Del(1)(-p)		Der(9)t(9;10)	
Der(1)t(1;3)		*Der(9)t(9;11)*	*3*
Der(1)t(1;12)		Der(9)t(9;18)	
Der(1)t(3;20)		*Del(10)*	*6*
Der(1)t(1;15)		*Der(10)t(10;15)*	*2*
Der(1)t(1;16)t(1;10)		Add(10)	
Der(1)t(1;8)	*2*	Der(11)t(10;11)	
Der(1)t(1;9)		Del(11)	
Del(1)		*Del(12)*	*2*
Der(1)t(1;18)		Del(12)(-p)	
Der(1)t(1;5)		Der(13)t(10;13)	
Der(2)t(2;5)		Der(14)(1;14)	
Der(2)t(2;6)		Der(14;15)	
Der(2)t(2;X)		Der(14)t(10;14)t(10;17)	
Del(3)	*2*	Add(16)	
Der(3)del(3)9q?)t(3;14)		Der(16)t(6;16)	
Del(4)	*2*	Der(16)t(6;X)	
Del(4)t(4;10)		*Dmin(17)*	*2*
Der(4)t(4;17)		Der(17)t(4;17)	
Der(5)del(5)(q?)t(5;10)		*Del(18)*	*2*
Der(5)t(5;10)		Der(18)t(18;X)	
Der(5)t(5;22)	*2*	*Der(18)t(7;18)*	*2*
Der(6)t(6;13)	*2*	Add(20)	
Del(6)	*2*	Der(20)t(18;20)	
Der(7)t(7;10)	*2*	*Der(X)t(16;X)t(17;X)*	*4*
Der(7)t(7;X)	*2*	*Del(X)*	*4*
Der(7)t(7;20)		Der(X)t(16;X)	
Der(7)t(7;21)		Der(Y)t(16;Y)	
Del(7)			
Del(8)			
Der(8)t(8;17)			
Der(8)t(8;22)	*5*		
Der(8)t(8;12)	*3*		
Der(8)t(8;19)t(19;21)			



5-1-1 Figure 5-1(a) Image of a single metaphase spread



5-1-2 Figure 5-1(b) Karyotype image of a different metaphase spread

The total number of chromosomal aberrations found in the six cells that were analysed by MFISH was approximately 120, of which simple chromosomal alterations were in the form of deletions, and were most commonly encountered in chromosome 10, which was deleted in all six (100%) cells analysed. This was followed, in the order of descending frequency, by chromosome 9, which was deleted in 5 out of six (83%) cells,

X-chromosome, 4 out of six (67%), chromosome 3, 2 out of six (33%), chromosome 4 (33%), chromosome 6 (33%), chromosome 12 (33%), and chromosome 18 (33%).
On the other hand, recurrent simple gains were observed only in chromosome 17, which, interestingly, had double minutes in two cells. In addition, three simple gains were observed, occurring only once in each case in chromosomes 10, 16 and 20.

The region on the array CGH of CHR 8, at approximately 38 Mb from p-ter, where the signal suddenly increases and continues to q-ter could possibly correspond to one of the breakpoints in the t(8;22) seen in the MFISH. Closer inspection suggests the boundary clones to be RP1-144M5 (at around the 34Mb position), RP11-155L11 (35Mb), RP11-98I12 (36Mb), RP1-197P20 (37Mb), (RP11-350N15 (38Mb), and RP11-44K6 (39Mb), so that genes within this region might possibly be contributory to a fusion protein with oncogenic potential. Although in such a very abnormal karyotype this must be a very tentative conclusion, it does illustrate a possible method to combine CGH microarray to MFISH results.

It was not possible in most cases to ascertain in more specific terms the chromosome arm of the donated segment in the region of translocation revealed by MFISH. Additional investigations would be required to characterise the breakpoints in both CHR 8 and 22 that are translocated and might possibly contribute to a fusion protein. Thus, at this stage of the study it is not possible to discuss the translocations in more specific detail with reference to genes that might be implicated. However it is of interest to notice that numerous translocations between chromosomes 8 and 22 have been reported in medical literature. A PubMed search found 242 entries (hits) although not all of them are limited strictly to the two chromosomes. Various combinations of translocation involving both arms of chromosome 8 and parts of chromosome 22 have been reported in association with a wide range of medical conditions. In some the diseases associated with various t(8;22) were not of the malignant kind, as for example, the association of constitutional t(8;22) with myasthenia gravis, leucocytosis and thrombocytosis (Keung et al., 2004). Other diseases associated with t(8:22) were related to haematopoietic and lymphopoietic-type malignancies (Demiroglu et al., 2001; Tasaka et al., 2002; Reid et al., 2003; Gupta et al., 2004) and solid lymphomas, in particular variants of Burkitt's lymphoma (Gerbitz et al., 1999; Yamamoto et al., 1998;

Rickert et al., 1999). In addition, there are occasional reports of translocations involving chromosomes 8 and 22 in solid tumours of other systems such as cardiac lymphoma (Klopfenstein et al., 1997), epithelioid sarcoma (Cordoba et al., 1994) and mixed mullerian tumour of the uterus (Sreekantaiah et al., 1999).

Probably the most characterised and documented t(8;22) is one involving 8q24 and 22q11, der(8)t(8;22)(q24;q11) which is observed in variant forms of Burkitt's lymphoma. This translocation is thought to implicate the C-MYC gene on 8q24 and immunoglobulin light chain, lambda, gene (Igλ) at 22q11 (Zeidler et al., 1994; Calasanz et al., 1997(a); Calasanz et al., 1997(b); Yamamoto et al., 1998). Another t(8;22) translocation that has been characterised involved fusion of the genes for breakpoint cluster region (BCR; 22q11.23) and epidermal growth factor receptor 1 (FGFR1; 8q11.2) in patients with chronic myeloid leukaemia (CML) (Demiroglu et al., 2001; Fioretos et al., 2001). It has been suggested that the t(8;22) in which the CMYC and light chain genes are fused, may contribute to oncogenesis in acute, mostly paediatric type malignancies (Gupta et al., 2004), while primarily promoting malignant progression in chronic haematogeneous malignancies (Yin et al., 2004). Thus, the translocation t(8;22)(q24;q11) is regarded as a marker of poor prognosis (Voorhees et al., 2004; Lones et al., 2004). Whether these various previously described translocations have any relationship to the translocations seen in the GBM/C1724 remains to be determined.

DISCUSSION

6-1 Overall aims

This thesis has been concerned with the detection and interpretation of copy number changes of the genetic material in astrocytomas. In general there are three types of large-scale chromosomal changes, which may provide useful clues to the mechanism of origin of a tumour and might also provide some indication of likely outcome. These include increase of material in a specific region, which might indicate amplification of an oncogene. Loss of a specific region may suggest the existence of a tumour suppressor gene. A truly balanced translocation between two chromosomes, which may for example result in the activation of an oncogene by the generation of a fusion protein will not be detectable as loss or gain of material. However, many translocations are not balanced (Krex et al., 2001; Jenkins et al., 2006; Mulholland et al., 2006) and in some instances, in theory copy number changes are detectable on one side of a translocation, delimiting a breakpoint. The changes which influence origin or progression of the tumour presumably arise as random events which confer some proliferative advantage and thus are selected and may come to dominate the tumour. However, some tumours develop genomic instability with each cell division generating new gross variation. It is not clear if the details of such variation can provide specific clues about origin or progression, but even in such situations an approach such as CGH will detect any copy number change present in the majority of tumour cells.

In this discussion I will assess the reliability of data obtained and consider the technical limitations of the metaphase approach. For the limited number of tumours further investigated by microarray, I will compare data obtained using the two approaches. I will then summarize, in table and diagrammatic form, the mass of data generated by this project and will attempt to relate the findings to previous work and to consider whether previous hypotheses are supported. Any relationship of CGH findings to age at diagnosis or length of survival after diagnosis will also be considered. It is not practical to assess the possible significance of all possible combinations of aberrations seen in the 32 tumours studied. I have therefore chosen to consider in some detail changes in chromosomal regions known to harbour genes

implicated in hereditary syndromes with which astrocytomas or other neurodevelopmental tumours are closely associated. I shall then consider genes known to be involved in astrocytoma progression (TSGs and oncogenes). Thereafter, I shall consider changes not previously reported but which are found in at least three independent tumours in this series. The discussion at this point will attempt to highlight genes with roles in neural development and established mutator phenotypes (caused by aberrations in genes for MMR).

The aim of this analysis is to generate additional hypotheses of pathways, genes and, indeed, different cell types which may be involved in the development of astrocytomas. These hypotheses might then suggest further work, which could identify biological features distinguishing different tumours and eventually provide tools for better prediction or treatment.

6-2 Reliability of metaphase CGH Results

The control experiments (reported in chapter 3) and the good agreement between the metaphase and microarray CGH results (excluding heterochromatic regions) on the seven tumours further investigated by this approach (chapter 4) give confidence that within the limits of the techniques used most of the results reported here are reliable. A discrepancy was the convincing loss of 16p in GBM/C1724, reported on metaphase but less convincing on microarray, and also 19p in /C1510, which is cytogenetically deleted in metaphase but not at all in array. Another small region of gain observed at 13q21 in the metaphase of tumour 1706 could not be seen on microarray.

Distal 1p, 19q, and 22, the three other GC-rich regions in which spurious profiles are known to occur due to labelling artifacts (Kallioniemi et al., 1994), could not be compared in the 7 tumours investigated by microarray CGH in my series since none had alterations in these regions in the metaphases. While these discrepancies are clearly important, at this level of the analysis their significance remains uncertain. It is interesting that a rather similar genomic microarray previously failed to detect known deletions in the TSC2 region, at 16p13, in two samples evaluated by this method (Ekong et al., 2004; Michalet et al., 1997; Sue Povey, personal communication).

Therefore, while the metaphase and array CGH data are discussed as such, in the thesis, the discrepancies and uncertainties have been kept in mind.

One finding, which needs further consideration is that metaphase CGH of two tumours did not reveal any chromosomal alterations in any of the autosomes, while 5 others revealed only one or two aberrations each, which is perhaps surprising for high malignancy grade astrocytomas. Possible explanations include failure to find small changes because of lack of sensitivity of the metaphase CGH technique, inappropriate choice of cut-off limits for reporting abnormalities, contamination of the sample by large proportions of normal DNA, and some genuine explanation such as methylation of critical genes, or oncogene activation by balanced translocations.

6-2-1 Limitations of the metaphase CGH Procedure

As mentioned in the introduction, metaphase CGH has a low resolution, capable of reliably detecting hemizygosity in deletions above 5-10 million bases (MB) (Van Roy et al 1997; Squire et al, 2001; Lupski, 2001). The threshold for detection of high copy number amplifications is better, estimated to be around 2.5 MB (Kearney, 2001). In terms of the procedures, CGH is technically demanding, requiring metaphase spreads of good quality, carefully controlled conditions for storage & handling of slides and adherence to optimum conditions during the labelling and hybridisation steps of the procedure (Karhu et al., 1997; Breen et al., 1999). Although computerized acquisition of images and karyotyping are facilitated by automated software packages, manual intervention is inevitable at various stages of the procedure. Manual intervention underscores the need for expert knowledge in the procedures in general, and introduces the possibility of human error especially at the level of karyotyping. Thus, technical issues can affect the outcomes of CGH though they cannot directly account for the absence of CNAs on chromosomes following a successful hybridisation.

The choice of the detection range, i.e., the cut-off limits used to generate ratio profiles of gain/loss across the axis of symmetry for the entire length of each chromosome, may be important. In this study the cut-off range for detection of loss and gain respectively was set at 0.8 – 1.2, and between 10 and 33 metaphases were examined for each sample. In some cases there were sections of borderline ratio profiles, i.e., the

situation when the ratio profiles are shifted so that the graph of ratio profiles and the cut-off limits are close but not actually intersecting so that copy number changes were not reported. In cases where large numbers of cells are examined, it might be reasonable to narrow the 'normal range', which increases the number of abnormalities reported. This approach was tested in several of the tumours with 'borderline' fluorescence graphs in this study. Narrowing the detection range to between 0.85 – 1.15 for tumours that did not show CNAs with initial cut-off limits, but demonstrated borderline ratio profiles, resulted in reporting of CNAs in previously borderline regions (see figure 6-1). However, data resulting from this alteration are not included in the overall results since ideally it would require adjusting the detection limits for the rest of the tumours as well. This would result in an enormous number of apparent CNAs of unknown significance. However for the detailed study of a particular tumour, or to answer a specific question especially if there is a possibility of contamination by normal cells, this could be very valuable.

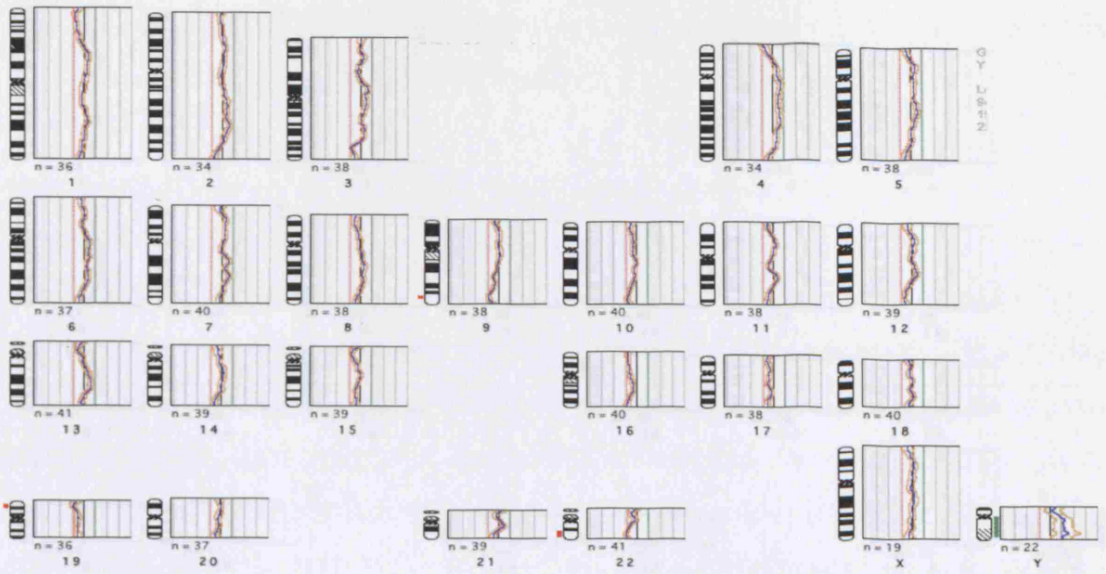


Figure: 6-1 Fluorescence ratio profile of AS24 (an anaplastic astrocytoma) with cutoff range set at 0.8-1.2.

In this CGH experiment 24 metaphases with a total of 881 chromosomes were analysed. There was one gain in the Y-CHR (Yp11.1-q12), and three losses located respectively at 9q34, 19p13.2-p13.3, and 22q12-q13. Although a candidate gene(s) at 19p has not been identified each of these chromosome regions is thought to harbour a gene or genes implicated in astrocytoma oncogenesis.

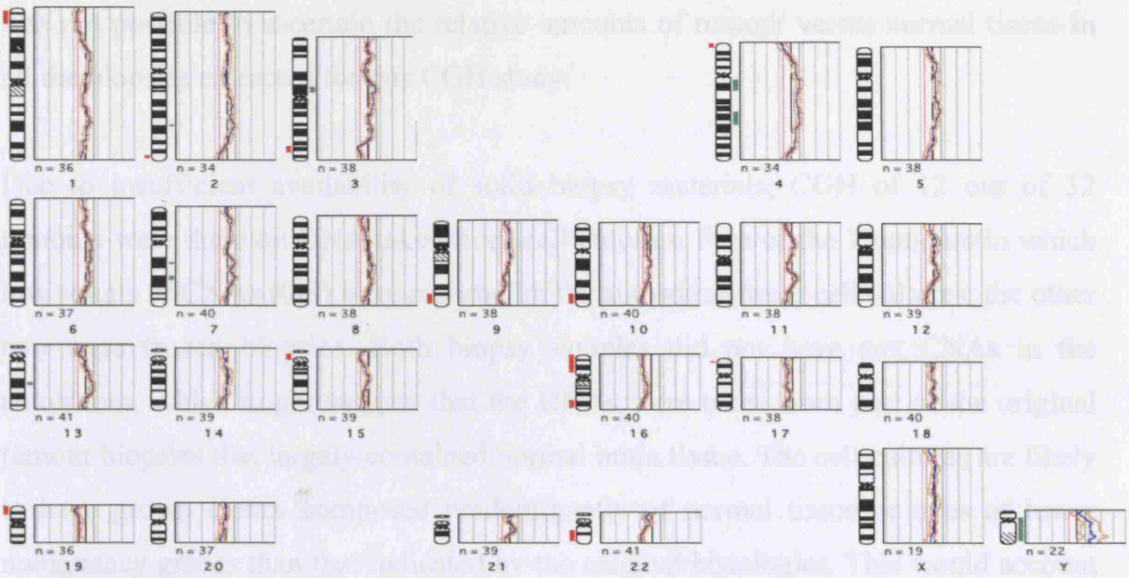


Figure 6-2: shows the difference in pick-up rate for CNAS caused by altering the cutoff range.

Figure 6-1 is generated from the same data as the one in figure 4-1(A) except that the cutoff range was narrowed down to 0.85-1.15, which increases the probability of detecting CNAs. As a result alterations have emerged in regions that were previously borderline, i.e., shifted in the direction of gain or loss without actually attaining the limits set for reporting aberrations. Thus, gains have now emerged at 3p12, 4q12-q13, 4q25-q28, 7q21, 7q31, 13q21, in addition to the original one at Yp11.12 (revealed under previous settings). Similarly, losses are clearly highlighted at 1p34.3-p36.3, 2q37, 3q26.2-q27, 4p16, 9q33-p34, 10q25-q26, 16p11.2-p13.3, 16q22-q24, 19p13.1-p13, and 22q11.2-q13

A quite probable reason for a lower-detection rate of CNAs in some of these tumours could be due to a relatively high proportion of normal - non-tumour tissue - in the biopsy sample. In order to gain meaningful information CGH requires at least 35% of tumour cells in the sample (Lichter et al. 2000). Although histological sections of all the samples were reviewed with a senior pathologist with the objective of confirming the diagnosis prior to DNA extraction, we relied on slides of previous histology sections because the majority of biopsy samples that were available for extraction were very small and could not be spared for a repeat, confirmatory, diagnostic procedure. Although clearly useful for ascertaining the original histological diagnosis, in reality archived histology sections do not necessarily reflect the status of the remaining 'tumour' specimen. After the original histology sections were taken, further sections of any remaining biopsy materials would normally have been taken for other investigative procedures such as immunohistochemical analyses etc. Thus, it

was not possible to ascertain the relative amounts of tumour verses normal tissue in all the biopsies extracted for this CGH study.

Due to insufficient availability of solid biopsy materials, CGH of 12 out of 32 tumours were done on DNA taken from cell cultures. Five of the 7 tumours in which low counts of CNAs (0-3) were reported in the autosomes were cell cultures; the other two were frozen biopsies. Both biopsy samples did not have any CNAs in the autosomes, which might suggest that the DNAs were taken from part of the original tumour biopsies that largely contained normal brain tissue. The cell cultures are likely to have grown clones composed predominantly of normal tissue or ones of lower malignancy grades than that indicated by the original histologies. This would account for the paucity of CNAs in the GBM solid biopsies UGBS2848m and PGBS2093, and the cell cultures PGBC160, PGBC1706, PGBC1752, PGBC2394 and PGBC2685. In three of the cell-lines cases PGBC160, PGBC1752 and PGBC1706) microarray analysis was possible and in all 3 cell-lines no clearly significant changes were found although there was some suggestion of increased overall signal for CHRs 5 and 19 in two of them. The existence of balanced translocations cannot of course be ruled out in these cases. In 3 of the other samples (/S2848, /C2394, and /C2685) there was insufficient material for microarray experiment. The array result on /S2093 and /C1510 are discussed below.

Microarrays of the tumours showing minimal changes on metaphase CGH indicated that 2 DNA sample had tumour material. PGBC1510, in which metaphase CGH showed apparent gains in 4 regions of CHR 7, was found by the array to have an increased signal all the way along chromosome 7. PGBS2093 revealed a strong signal of gain in the EGFR region and underrepresentation of CHR 10, both of which were undetected by metaphase CGH. These array findings suggest that unlike the first three, which may have grown a disproportionate amount of normal tissue, PGBC1510 and PGBS2093 certainly had some amount of tumour DNA.

Finally, it could be argued that tumours, from the low-CNA-count experiments, originated from epigenetic mechanisms such as altered promoter methylation rather than other conventional mechanisms like loss of heterozygosity (LOH), which would be characterized by deletions and amplifications. Epigenetic events are clearly

important in astrocytomas of low- and high malignancy grades (Costello et al., 2000; Debinski et al., 2003; Nakamura et al., 2005). In addition, there are reports in the literature of findings of high-level chromosomal instability in the absence of structural alteration (Doneda et al., 1998). Presumably, epigenetic mechanisms, acting as the primary cause to the exclusion of other mutational mechanisms, would be highly unlikely in astrocytomas that have attained a high malignancy grade such as the ones in this study. Therefore, finding normal CGH in astrocytomas of a high malignancy grade would be highly suspicious (Denise Sheer, personal communication). In this study, it seems most likely that the cells of three tumours examined were not entirely representative of the original tumours, and although these tumours have been kept in the analysis, where appropriate, this doubt has to be kept in mind.

6-3 Overall results

This study, which looked at 32 high malignancy grade astrocytomas using comparative genomic hybridisation found DNA sequence copy number alterations in 30 of them. Figure 6-3 gives a visual overview of the locations of the entire panel of DNA sequence CNAs in each arm of the chromosomes, while Table 6-1 gives a numerical view of the distribution of CNAs in 26 informative tumours, providing, in digestible form, the relative copy number changes on each arm of the chromosomes. A detailed list of all CNAs revealed in each tumour is presented in Appendix 1.

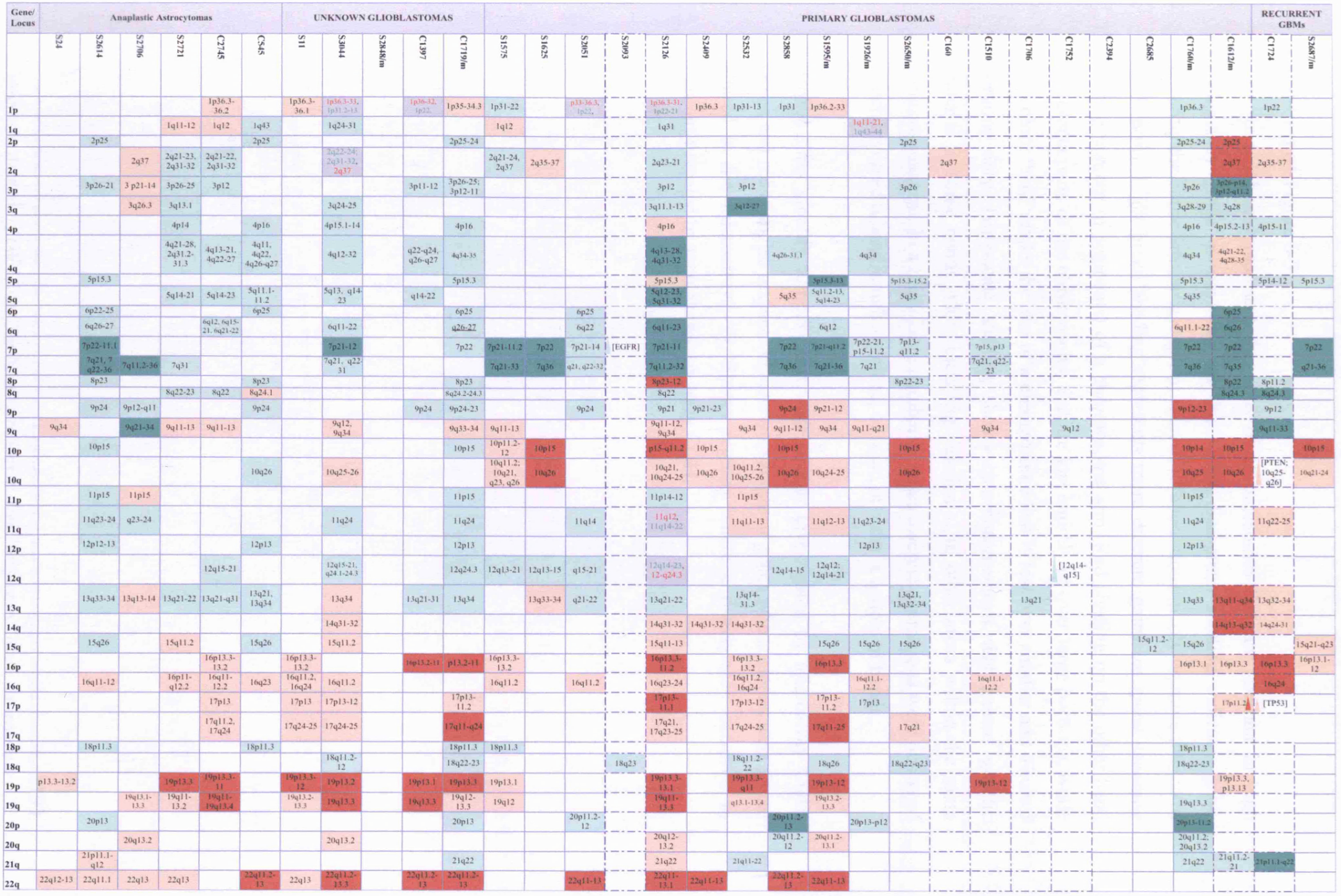


Figure 6-3: Panel of CNAs in all 32 tumours

Caption of Figure 6-3:

The shaded boxes represent the arms of chromosomes that are aberrant in high malignancy grade astrocytomas in this series. Sea-green shading indicates gains covering the entire arm, or a major portion of it (aberrations involving more than ½ of CHR arm). Light green shading represents more discrete gains (or gains spanning limited number of segments) of a chromosome arm. Red shading on the other hand represents parts of the chromosome that are lost entirely or over large portions, while the colour "tan" marks the arm of a chromosome that is lost over smaller and discrete portions only. Gray-scale shading shows chromosome-arms that gained genetic material in some bands while incurring deletion(s) in other areas within the same chromosome arm. In a fairly large number of cases discrete aberrations were observed which involved a band, small segments of it, or, at most, a couple of bands.

Broken lines identify tumours that were further investigated by microarray CGH. Green and red triangles respectively show a sample of genes whose clones were found altered in the microarrays. /§, Shows regions where a discrepancy occurred in findings revealed by metaphase and array CGH.

Table 6-1: Distribution of DNA Copy Number Aberrations in metaphase CGH of 26 informative tumours

	CNAs (Whole)							Number of CHR bands in CNAs		
	Gains			Losses			Total No of CNAs	Gains	Losses	Total
Chr. No	p-arm	q-arm	Total	p-arm	q-arm	Total				
1	10	4	14	11	1	12	26	55	103	158
2	6	6	12	1	6	7	23	84	76	160
3	10	5	15	1	1	2	27	117	6	124
4	7	9	16	1	1	2	26	123	20	143
5	7	9	16	1	1	2	22	105	2	107
6	5	7	12	0	1	1	16	148	15	163
7	14	16	30	0	0	0	24	319	0	319
8	6	7	13	1	0	1	12	102	8	110
9	10	3	13	3	11	14	26	97	68	165
10	2	0	2	10	12	22	24	4	156	160
11	4	8	12	3	5	8	20	36	46	82
12	6	9	15	0	2	4	14	68	11	68
13	Acro	2	12	Acro	6	6	21	65	61	126
14	Acro	0	0	Acro	7	7	8	0	71	71
15	Acro	5	5	Acro	4	4	5	2	13	15
16	0	0	0	14	10	24	21	0	112	112
17	1	0	1	8	8	16	21	1	80	81
18	5	6	11	0	0	0	12	32	0	32
19	0	1	1	13	10	23	21	4	145	149
20	6	2	8	0	4	4	13	25	12	37
21	Acro	6	6	Acro	1	1	7	30	2	32
22	Acro	0	0	Acro	12	12	12	0	80	80

Explanation for Table 6-1

The columns labelled 'CNAs (Whole)' in table 6-1 show the total number of discrete abnormalities recorded by the software in each composite metaphase images for the 26 informative tumours. They range in size from one band (or less in some cases) to a whole chromosome. The columns for 'number of CHR bands in CNAs' show the total number of bands (G-banding pattern at the 850 band resolution (NCBI map viewer)) altered. The abbreviations: Acro, acrocentromere region

Continuation of text...

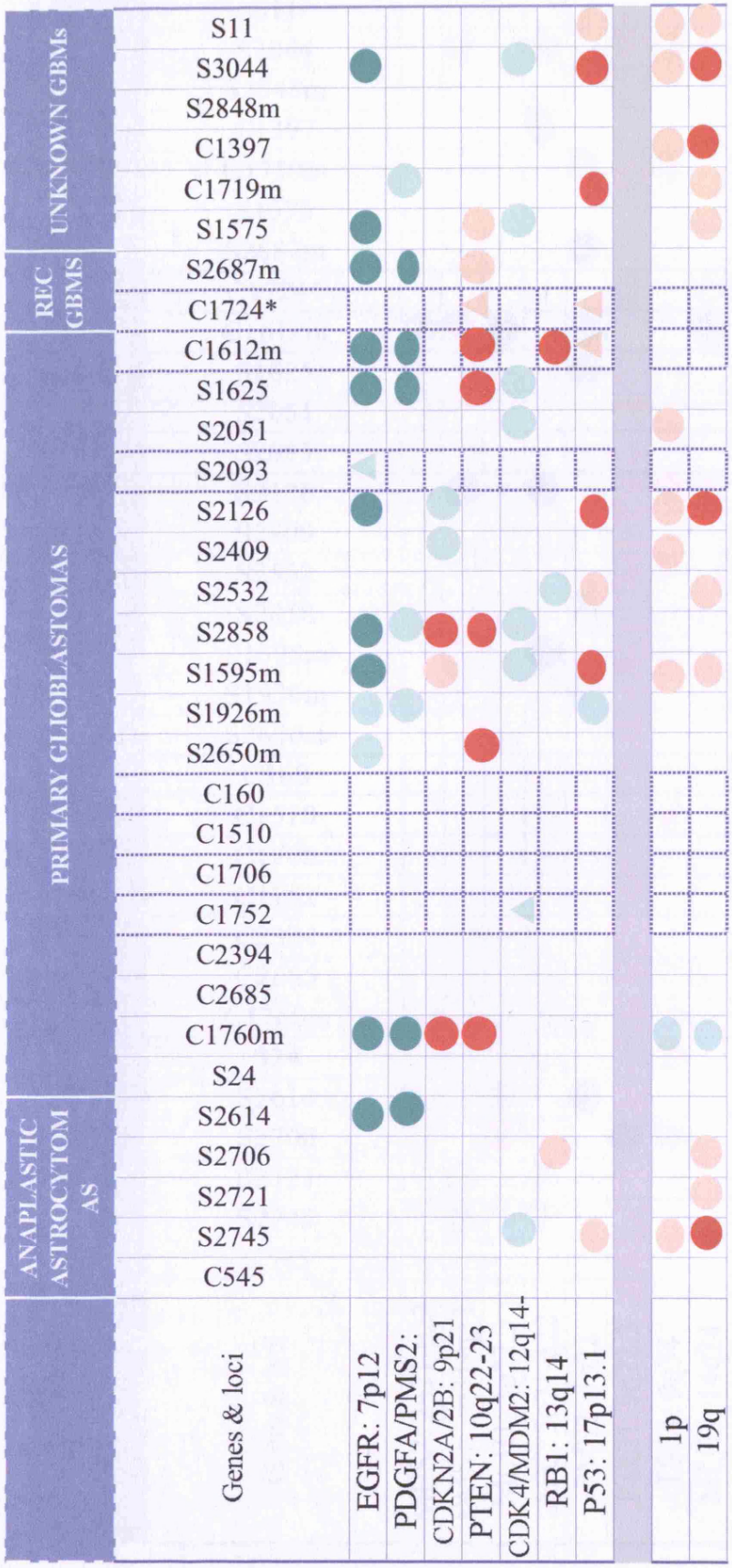
From table 6-1, the pattern of CNAs observed in chromosomes known to harbour genes with roles in pathogenesis of astrocytomas, for example gains of 7 and 12, and loss of 10, is confirmed. Both arms of CHR 7 were only gained, with no losses detected by metaphase CGH, while CHR 10 was predominantly lost, with only two instance of gain on 10p. The not-so-well described alterations such as gain of 18, 4 and 5, and loss of 16, 19, and 1p are reported with relatively high frequencies in this study. Interestingly, based on the metaphase data alone, CHRs 9, 13 and 17, with well-described deletions at 9p21, 13q14 and 17p13.1, respectively, are apparently altered with much lower frequencies in this series.

However microarrays of one tumour in which metaphase CGH reported no alteration in regions of 10q and 17p, revealed alterations of clones with sequences matching those of PTEN and TP53 respectively, while another tumour had amplification of EGFR. This clearly demonstrates the limitations of the metaphase CGH approach for detecting subtle DNA sequence copy number alteration that may be involved in pathogenesis of astrocytic tumours. However the high resolution of microarrays does also bring problems (especially in the absence of blood DNA from the patient) because of the very large number of 'normal' copy number variants now known to be present in the human genome (see for example Redon et al 2006) which are much more likely to be detected by this approach. This is not such a difficulty when there is a strong prior hypothesis (as in the cases above) but for any new findings, the possibility of normal polymorphism has been kept constantly in mind in this study

The number of chromosomal abnormalities that have been detected by CGH in astrocytomas shows a progressive increase with the malignancy-grade of a tumour (Kleihues and Cavenee, 2000), and certain alterations occur more frequently in association with tumour-grade (Von Deimling et al., 1995; Ohgaki, 2005). This observation formed the basis for the prevailing models for astrocytoma progression pathways. In this study (see table 6-1 and figure 6-3) the CGH data of 26 informative tumours found DNA copy number alterations in all chromosomes although this was variable, with some more affeted than others. In general the number of aberrations observed in cell lines tended to be more than in solids. Aberrations, which are well established for astrocytomas and where at least some of the responsible genes have been identified, were frequently seen in this series (see figure 6-4) and will be discussed later. Deletions at 1p and 19q, which occur more frequently, and are of prognostic significance in oligodendrogliomas (Cairncross et al., 1998) and some astrocytomas (Reifenberger G and Louis DN, 2003), were also observed in this study and are also recorded in figure 6-4. The aberrations in loci implicated in syndromes with susceptibility to astrocytomas are shown in fig 6-5.

In addition, the study revealed novel locations of recurrent copy number aberrations (defined as occurring at least three times in this series). Some of the affected regions were found to harbour loci of genes with roles in CNS development, some of which are implicated in malignancies of other systems, and some involved loci of genes known to be responsible for established mutator phenotypes. However, in most cases the aberrant regions are gene-rich, which, given the limits of CGH, makes it difficult to implicate a particular gene, or conversely to eliminate other probable genes of interest (GOIs) without supplementary investigations.

6:4 Distribution of CNAs at loci of genes with established roles in sporadic astrocytomas



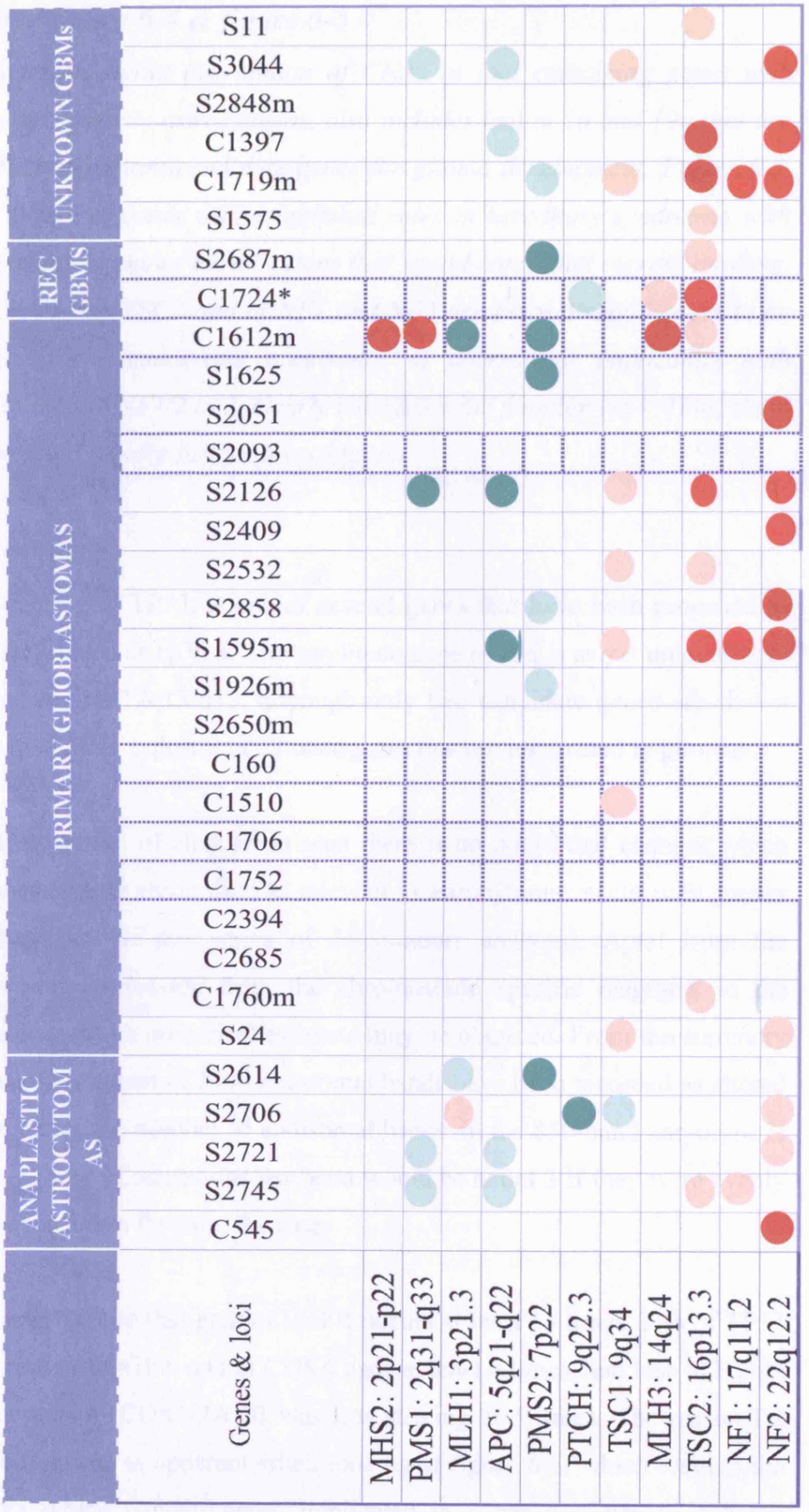


Figure 6-5 Distribution of CNAs at loci implicating genes with established roles in astrocytoma-associated syndromes, but not established in sporadic astrocytomas

Interpretation of figure 6-4 & figure 6-5

The figure 6-4, which shows distribution of CNAs at loci containing genes with established roles in sporadic astrocytomas, also includes loci at 1p and 19q that are thought to harbour important candidate genes for glioma development. Figure 6-5, showing CNAs at loci of genes with established roles in hereditary syndromes with astrocytoma association, shows combinations that would contradict current thinking. Aberrations of TSC1 and TSC2 and of NF1 and NF2 are respectively thought to be mutually exclusive. The concurrent occurrence of aberrations implicating both TSC1/2 in 4 tumours and NF1/2 in 2, clearly contradict the popular view. Thus, these curious findings would require further investigation.

Continuation of text...

TP73, a close homolog of TP53, is one of several genes that have been proposed as possible candidate located at 1p36.3. The candidate gene at 19q is as yet unidentified. In the case of the region 12q13-q15, although only two candidate genes are shown here it is known to contain a cluster of 12 oncogenes that are implicated in gliomas.

Although a wide spectrum of changes is seen there is no doubt that changes which must include genes already recognised as relevant to astrocytomas occur with greater than random frequency in this series of 26 tumours analysed. Apart from the overwhelming visual impression from the chromosome specific diagrams in the results section a very rough numerical estimate may be obtained. From the summary table 6-1 it is clear that a total of 2494 autosomal bands have been recorded as altered in these tumours. Since the number of autosomal bands on the 850-band karyotype is 799 the average number of alterations per band would be about 3 if they were evenly distributed. However this is far from the case

From figure 6-4 one can see that gain of EGFR occurs at least 13 times, loss of TP53 at least 9 times, gain of PDGFA and of CDK4 each at least 8 times, and loss of PTEN also 8 times. The loss of CDKN2A /B was less striking at 5 times. The reason for mentioning these numbers is apparent when looking at figure 6-5, which records the alterations in loci which contain genes implicated in syndromes which included astrocytomas, excluding those already reported to be important in sporadic

astrocytoma, such as TP53. A conservative interpretation obtained by combining information from fig 6.5 and the detailed table of karyotypes shows some loss of TSC2 in at least 12 tumours, NF2 in at least 12 tumours, TSC1 in at least 7 tumours and APC in at least 6 tumours. The frequency with which loci of astrocytoma susceptibility genes are implicated would suggest the probable involvement of several of them in the pathogenesis of sporadic astrocytomas. In addition, altogether 25 out of 32 tumours in the study (GBM/C1724 and GBM/C1724/mda are the same tumour) had aberrations implicating at least one of the 19 loci considered in figure 6-4 and 6-5

6-4 Conclusions from overall preliminary analysis

These data have led to several observations a number of which are in agreement with previous studies while others apparently do not. For example,

1. The frequent gains in copy number at CHRs7p and 12q, possibly implicating EGFR at 7p12 in 13 tumours, PDGFA at 7p22 (in 7 tumours) and MDM2 at 12q14-15 (in 9 tumours; 28%), would be in agreement with other studies that have found these to be the oncogenes most commonly amplified in astrocytomas. The relatively high frequency of copy number losses in 10q (>70%) is also in agreement with other studies
2. The copy number losses at 1p and 19q, and the correlation between the two in a number of the tumours, are also in agreement with other studies.
3. Although DNA copy number losses in several tumours implicated CDKN2AandCDKN2B (9p21), RB1 (13q14) and TP53 (17p13), respectively, the relative frequencies observed in this study appear lower than reported by other studies
4. Several uncharacteristic alterations were recorded in a significant number of the tumours. In particular, amplifications occurred at several loci implicating established TSGs at 9p21, 9q34, 13q14, 17p13, and at a number of loci implicating MMR genes.
5. A major difference with other studies seems to be in the distribution of DNA copy number alterations at loci implicating genes with established roles in syndromes with astrocytoma associations. Approximately 80% of the tumours

(26 out of 33) had copy number aberrations involving loci of one or more of the 14 syndrome-associated genes.

Thus, these data appear to suggest that some of these genes could influence development of sporadic astrocytomas. This is surprising, and could be highly controversial in view of past evidence against them (for example, Rey et al., 1992; Green et al., 1994; Rubio et al., 1994; von Deimling et al., 1994; Hoang-Xuan et al., 1995; Ng et al., 1995; Henske et al., 1996; Wienecke et al., 1997; Parry et al., 2000; Wimmer et al., 2002; Lau et al., 2003). Although several of these authors found LOH in some astrocytomas, they were unable to find mutations to confirm their findings. Hence, the need to explain the findings in this current study inevitably dominates parts of this discussion.

To answer the issues raised, I will discuss the findings under several subheadings. 1) First, an attempt will be made to correlate the findings with pathways that have been proposed for oncogenesis and progression of astrocytomas. Bearing in mind the limitations of the technique, this will give an idea whether the cytogenetic data for each tumour support the clinical category (whether a primary (de novo) or secondary glioblastoma) of the respective tumour. 2), A discussion of aberrations at 1p, 19q, the two established loci for which no genes have as yet been confirmed, will then follow. Studies, especially of oligodendrogliomas and low-grade astrocytomas (Buckner et al., 2003; Jenkins et al., 2006), have established associations between CNAs at these two regions with patients' response to therapy and prognosis in general. Any possible correlation between CNAs in these regions with survival in this series will be considered here. The last part of the discussion in this section will address issues underlying CNAs at loci implicating syndromes with astrocytoma associations.

6-5 Correlation of DNA copy number alterations with prevailing tumorigenetic pathways

Tumours investigated in this study will be divided into several categories based on the number of aberrations implicating 7 genes known to be involved in the pathogenesis of sporadic astrocytomas, starting with those with few (or no) alterations, and ending with the ones in which the most number of alterations (per tumour) were recorded.

6-5-1 Tumours with apparently no CNAs implicating genes with established roles in sporadic astrocytomas.

Ten (31%) tumours did not reveal CNAs in any of the 7 loci of genes with established roles in development of sporadic astrocytomas. Five of these had no CNAs on metaphase and the concerns about the amount of DNA present have already been noted. Still, the isolated CNAs revealed by the metaphase in /C1706 and /C160 are of interest as several other tumours showed discrete alterations at similar loci. For example, the discrete copy number gains at 13q21, in /1706, was also observed in 8 other tumours in this series, while the equally discrete loss at 2q37 found in /C160 was shared by 6 other tumours. However these aberrations (in the metaphases) were both not corroborated by the microarrays, so the possibility that these are artifacts cannot be excluded. However, the roles of potential candidate genes mapped to these aberrant loci are discussed in detail later under "novel alterations".

The other 5 tumours in this group, which were thought to be genuine tumour material each had between 3-18 CNAs. Of the five, only GBM/C545, remarkably, had all 16 CNAs at novel loci, while the other 4 had between 1-3 CNAs at loci implicating genes with established roles in syndromes with astrocytoma associations. For example two of the tumours apparently lost in the region of TSC1 (AA/S24 and GBM1510), and one at TSC2 (GBM1397). This group is potentially of great interest but would need much more investigation before any firm hypothesis could be proposed, especially as some of the findings were not confirmed on microarray.

6-5-2 Tumours with CNAs implicating genes with established roles in development of sporadic astrocytomas.

22 tumours had CNAs at one or more of seven loci implicating genes with established roles in development of sporadic astrocytomas (DSA), i.e., EGFR, PDGFA, CDKN2AandCDKN2B, PTEN, CDK4/MDM2, RB1 and TP53. In 3 of these tumours (GBMs /C1752, /S2093, and /C1724) the only CNAs implicating these genes were revealed by array, which metaphase CGH had failed to detect. DNA copy number gains were revealed at loci of CDK4/MDM2 and EGFR, in /C1752 and /S2093

respectively, while losses of PTEN and TP53 were found in /C1724. This clearly demonstrates the superiority of the array over metaphase CGH method, a fact that has been kept in mind throughout the discussion of these data. In this small group of tumours, except for these alterations the tumours might not have fitted in the category of sporadic astrocytomas. The first two of these tumours, along with several others in which fewer than 3 CNAs were found in the autosomes, have already been discussed In the section that follows, each of the other tumours is discussed separately.

6-5-2.1 GBMs/C1612 and 1724

GBM1612 and GBM/C1724 are both from the same patient, 1612 being the first biopsy and 1724 being a recurrence after an unknown time. Since the patient only lived eight months after the diagnosis it could not have been longer than this. 1612 did not survive as a cell-line and the amount of DNA available was small, so whole genome amplification was used. 1724 was studied as a cell-line. Both tumours were studied by microarray and 1724 also by M-FISH. Both lines showed extensive changes with some features in common, in particular a substantial gain in signal from a 4 MB region on 4p, which was also seen to a lesser extent in 5 other samples

Most CNAs in PGBMC1612m involved extensive segments, for example, the whole of CHR 2, 10, 13q and 14q were under-represented while 3q, 6, 7, and 8 were gained. However, 3 regions of discrete gains were at 3q28, 4p15.2-13 and 21q11.2-21 while discrete losses were located at 4q21-22, 4q28-35, 11q22-25, 13q32-34 and 14q24-31 and 17p13. The losses in the loci of RB1, PTEN and TP53, and gains corresponding to EGFR would fit in the category of primary GBM - based on existing pathway models, and is in agreement with clinical data on this patient. However the additional gain corresponding to PDGFA (or PMS2) and MLH1, as well as deletions corresponding to MSH2 and TSC2 would point to a possibility of PGBC1612 being a secondary GBM while a probable role of these genes in sporadic primary GBMs would raise issues about the role of genes hitherto not believed to have roles in this category of tumours. The microarray data on 1612 in general showed good agreement with the metaphase data, although the specific clones containing the PTEN gene were seen on the microarray to be virtually totally deleted, with much less signal than the rest of CHR 10.

RGBC1724 was shown by microarrays to have striking loss of PTEN and the region of TP53 on chromosome 17p. Other features of 1724 showed some similarity to 1612, such as the identical gain on 4p already mentioned, and losses on 13q and 14q, but also some differences in whole chromosome balance such as the lack of gain of chromosome 7. In at least two cases (chromosome 8 and chromosome 11) the microarray data on 1724 but not on 1612 showed a discontinuity suggesting a possible breakpoint. Examination of the M-FISH results on the 1724 cell-line provided a rather startling explanation for the probably non-stability of the whole chromosome balance, as the karyotype showed approximately 300 chromosomes per cell and the six cells examined were all so different. It was interesting that there was an 8;22 translocation in five of the six cells, which might well have reflected the possible breakpoint in the region of uncoordinated centrosome (movement)-5D (UNC5D) between clone RP11-144M5 and RP11-155L11 seen on the microarray. Another translocation seen in 4 cells could have implicated the possible break on chromosome 11 in the region of Fanconi anaemia, complementation group F (FANCF) (postulated to be a pseudo gene – NCBI Aceview) between clones RP11-34N19 and RP11-72C9.

It was impossible to see the position of the extra 4p material on the MFISH, which was rather surprising. Although it would be of interest to clone the breakpoint on the 8;22 translocation the very large number of chromosomes make further study and interpretation of this line quite challenging

Of the 3 tumours found to have only one aberration at loci implicating established genes with roles in development of sporadic astrocytomas, AA/S2706 had loss at the locus of the RB1 gene; GBM/S2051 had gains implicating CDK4/MDM2, while GBM/S11 had deletion implicating the TP53 gene. GBM/S2051 was a primary GBM, but no data were available on the category of GBM/S11.

6-5-2.2 AA/S2706

AA/S2706, showing loss in the region of RB1 was additionally deleted at 7 novel loci with 22q13 and 19q13.3-13.1, (respectively deleted in 12 and 10 other tumours) being

the most frequently deleted in the study. The loss of copy number at 2q37, shared by 5 other tumours, has already been highlighted. This tumour was also deleted at 20q13.2, which it shares with 3 others in this series. These frequently deleted novel loci may harbour important TSGs. In addition, AA/S2706 had 5 regions of novel gains; the large gain spanning 7q11.2-q26 was common to 9 other tumours, while 5 others had discrete amplicons at 7q22, q23, and q31, which might point to loci of important oncogenes in the long-arm of chromosome 7. Another frequent region gained by AA/S2706 was 11q23-24, a discrete region of novel gain, shared by 7 other tumours in this series.

6-5-2.3 GBM/S2051

In addition to the gain at 12q12-14, GBM/S2051 lost at 1p36.3-33 (which was also observed in one other tumour) with 7 other tumours sharing the discrete loss of 1p36. This tumour additionally lost at 2 of the most frequently deleted loci in this study, 16q11.2 and 22q11-13. Although the 16q11.2 region is heterochromatic and polymorphic, this finding is recorded here and in other tumours because there are genes of possible significance (e.g., RBL2). There were additional gains in 10 novel regions, the 2 most frequent discrete regions gained being 1p31-22 and 13q21-22 that are shared by 8 other tumours. Other discrete regions of novel gains were at 12q15-21, which was shared by 6 tumours, while 6p25 and 9p24 were each shared by 4 tumours. Losses at 1p and NF2 do not fit the pattern for primary GBMs as proposed in existing pathways model. Although the clinical data indicated that GBM/S2051 was a primary GBM, the cytogenetic data suggest that this tumour could pass for a secondary GBM, an optic pathway- (Dasgupta et al., 2005) or other glioma in another cerebral location, typically arising in association with neurofibromatosis type 2. Otherwise, like in 1724 the data raise issues concerning the probable roles of CNAs at loci of genes implicated in syndromes with astrocytoma association, in development of sporadic primary glioblastomas.

6-5-2.4 GBM/S11

GBM/S11 (with the loss implicating the TP53 gene) additionally had losses at loci implicating the TSC2 (in common with 11 other tumours), 1p36 (shared by 7 others) and 19q13.2-13.3, which was deleted in 10 other tumours, 5 of them deleted in the

entire q-arm. Remarkably, there were no gains revealed in UGBC11, but there were 5 regions of novel deletions, with 4 of them being among the most altered in this series. The deletions of 16q11.2 and 19p13.3-12 have already been highlighted in other tumours. Other novel deletions were at 16q24 (shared by 4 other tumours), 17q24-25 (by 7 others) and 22q13 (distal to the NF2 locus) (shared by 12 other tumours, of which 9 had deletions spanning the entire 22q-arm). These novel regions could harbour important TSGs. There was no clinical data on the category of this tumour however the pattern of CNAs at established loci would fit the pathway to secondary GBM. This combination of CNAs is shared by GBMs of oligodendroglial origins (Cairncross et al., 1998).

6-5-3 Tumours with 2 CNAs at loci of genes for development of sporadic astrocytomas

6-5-3-1 AA/S2745

This tumour had increased copy number at the CDK3/MDM2 locus and loss at the locus of TP53, which fit in with the secondary pathway to GBM. In addition, there were loss of copy numbers at the loci of NF1 and TSC2, genes with important roles in astrocytoma-associated syndromes, and combined loss of 1p36 and 19q. The gains at 2q31-q33 and 5q14-q23 could imply that the usual mismatch repair genes located in the respective loci might be involved through somewhat different mechanisms in the pathogenesis of AA/S2745. Alternatively there could be different oncogenes mapped in these regions that may have contributed to pathogenesis of this anaplastic astrocytoma. This tumour also had 7 regions of novel copy number gain, and a similar number with loss. Novel gains were at 2q21-22 (shared by 4 other tumours), 3p12 (5 others), 4q (with discrete gains at q13-21 and q22-27, respectively shared by 5 others), 6q (with discrete gains at q12, q15-21 and q21-22, respectively shared by 5 tumours) and 8q22 (5 others). However, the 2 regions that were most frequently gained were at 12q15-21 (distal to the CDK4/MDM2 locus) and 13q21-q23, which were respectively shared by 7 and 8 other tumours in this series. Any of them could harbour important oncogenes. Novel losses were at 1q12 (lost in 3 other tumours), 9q11-13 (lost in 6 others), 16q11-12.2, 17q11.2 (3 others), 17q24 (6 others), 19p13.3-11, and 19q11-13.4, most of which have already been highlighted.

6-5-3-2 AA/S2614

This AA gained at 2 regions implicating genes with established roles in sporadic astrocytomas, and at the same time there was one gain at the MLH1 locus. There were no losses at any of the regions of a TSG with established roles in sporadic astrocytomas or syndromes with astrocytoma associations. The only losses were at novel loci: the pericentromere heterochromatin region, 16q11-12 (lost in 12 other tumours), 21p11-q12 (only loss in the study) and 22q11.2. There were 14 regions of novel gain a number of which were also frequently gained in other tumours. However, gains at 7p12 and 7p22 suggest involvement of EGFR, at the former locus, and either PMS2 or PDGFA (or both?) at the latter locus. Although EGFR and PDGFA have both been found amplified in glioblastomas, it is believed that EGFR is predominantly amplified in primary as opposed to secondary glioblastomas in which PDGFA is thought to be the more likely to be amplified (Reifenberger et al., 1996; Weber et al., 1996). The gain of the locus for EGFR in AA/S2614 suggests that both genes may be amplified in a subgroup of secondary glioblastomas. Alternatively, it might be PMS2 rather than PDGFA that is amplified at 7p22.

6-5-3-3 GBM/S2650

PGBS2650 had CNAs at 2 loci of known astrocytoma genes – a gain of 7p12 (EGFR), and deletion of 10q, corresponding to the locus of PTEN. This combination is considered as the classical representation of the route/pathway to primary or de novo glioblastomas (Kleihues and Cavenee, 2000). Indeed, GBM/S2650 was one of only 6 tumours (of which 5 were due to insufficient tumour-DNA in study samples) in this study that did not have alterations in any of the loci implicating genes typically associated with astrocytomas-related syndromes. This tumour, in addition, had 9 regions of novel gains and 2 of loss. The gains were at 2p25 (seen in 4 other tumours), 3p26, 5p15 and 13q32-34 (each gained in 5 others), 8p22-23, 15q26, 18q22-23 (each gained in 4 other tumours), 5q35 (3 others), but the most frequently gained region was 13q21 (already highlighted) that was shared by 8 other tumours in this study. The 2 regions of novel loss were 10p (lost in 8 tumours – with 2 tumours showing discrete losses respectively at 10p11.2 and 10p15), and 17q21 in which 3 other tumours were also deleted. These novel regions of copy number changes may harbour oncogenes and TSGs with roles in development of primary GBMs.

6-5-3-4 GBM/S2532

GBM/S2532 had loss at 17p13-12, implicating the TP53 gene, with gains at 13q14-31.3, spanning the RB1 locus. Two discrete losses at 9q34 and 16p13.3-13.2 respectively implicated TSC1 and TSC2, which might imply an association with tuberous sclerosis type 1. Thus, despite losses corresponding to the TP53 locus, GBM/S2532 does not conform to any of the prevailing astrocytoma progression pathways. There were 6 additional regions of gain and 9 of loss, most of them at novel locations that were frequently altered in other tumours. The most frequent gains were at 13q in which 11 other tumours gained at 2 discrete loci (13q21-22, gained in 8 other tumours, and 13q33-34, gained in 5 others), and 1p in which 7 other tumours gained, 4 of them within a small locus at 1p22. Other than losses at 16q11.2, 19p and 19q that have already been highlighted, other frequently deleted novel loci in this tumour were at 10q25-26 in which 10 tumours were deleted with 5 spanning this locus, 14q31-32 (also lost in 5 others) and 17q24-24 that was deleted in 6 other tumours. These frequently altered loci may harbour important oncogenes and TSGs respectively.

6-5-3-5 GBM/S2409

This tumour gained at 9p21-23 (which was the only gain recorded), and lost at 1p36 and 22q11-13. The gain of 9p21-23, spanning the CDKN2A/2B locus at 9p21, is unusual since this locus is usually deleted in high grade-astrocytomas. However the losses at 1p and 22q could implicate TP73 and NF2 respectively. There are in addition 3 regions of novel loss – all discrete deletions, at 10p15 (which is spanned by 7 other tumours), 10q26 (similarly deleted in 3 other tumours, with 5 others having larger/spanning deletions), and 14q31-32 (similarly discretely deleted in 4 other tumours with a fifth tumours spanning). These interesting regions may harbour important TSGs. Clinical data suggested that this tumour was a primary GBM. The gain at 9p21 might suggest a different mechanism of involvement of this locus in this astrocytoma or the presence of an entirely different candidate oncogene in this region. The combination of -1p and -22q in pathogenesis of this tumour would suggest involvement of the pathway to secondary glioblastomas.

6-5-3-6 GBM/C1719m

GBM/C1719m gained in only one locus of genes with established roles in astrocytomas. The discrete gain at 7p22 could implicate either PDGFA or PMS2 (or both) and a deletion at 17p13 possibly implicating the TP53 gene. These were the only aberration implicating any of the genes with established roles in sporadic astrocytomas. However, cytogenetic analyses found alterations, all of them deletions in 3 regions with established astrocytoma associations. The losses at 9q34, 17q11.2 and 22q12.2 could implicate TSC1, NF1 and NF2 genes all of which have established roles in syndrome with which astrocytomas are associated. In addition, there were 20 regions of novel gains and 3 of losses. No clinical data could be found on the tumour category for GBM/C1917/m. The alterations in region of genes with established roles in astrocytomas could place GBM/C1719/m in the category of secondary GBM.

6-5-4 Tumours with 3 CNAs at loci implicating genes with roles in development of sporadic astrocytomas

6-5-4-1 GBM/C1760m

GBM/C1760/m, if considered based on the loss of 10q and 9p, respectively spanning the loci of PTEN and CDKN2AandCDKN2B, and gains corresponding to EGFR, would fit in the category of primary GBM. This would be in agreement with clinical data on the category of this tumour. However, the amplification PDGFA or /and possibly PMS2 could place it in the category of secondary GBMs. Presumably it would be in the subgroup of astrocytomas with concurrent EGFR and PDGFA amplification. This tumour has additionally gained the 1p36 region and 19q13.3 and has loss at the 16p13 locus, which could implicate TSC2 in its pathogenesis. There are additionally 18 regions of novel copy number gain and 2 of loss, the latter being at 16q11.2-22 and 10p.

6-5-4-2 GBM/S1926m

GBM/S1926 had discrete gains at 7p12 and 7p22, with the possibility of involvement of PMS2 or PDGFA in the latter locus. Uncharacteristically, it also had a discrete gain at 17p13, which could suggest a gain-of-function aberration at the TP53 locus. There were no losses in any of the regions of genes with established roles in astrocytomas,

irrespective of category (whether sporadic or associated with established hereditary syndromes). Thus, the distribution of CNAs in this tumour does not correspond with any of the existing model pathways for progression to GBMs. This would suggest that alternative pathways might exist. However, there were 8 additional regions of novel gains and 3 of loss. The 3 regions of novel loss were at 1q11-21 (seen in 3 other tumours), 9q11-21 (6 others), and 16q11.2-12.2, a discrete region that is deleted in 10 other tumours in this study. One or more of these novel regions of loss could harbour TSGs that may have contributed to pathogenesis of this GBM.

6-5-4-3 GBM/S2126

This tumour had the largest number of aberrations in the study. Almost all chromosome arms were affected except 2p, 6p, 12p, 18 (p/q), and 20q. The combinations of CNAs -1p, +PMS2, +APC, +CDKN2A, +12q, -PTEN, -TSC2, -19q and –NF2 would more likely suggest the possibility of this tumour being a secondary GBM. In fact, clinical data obtained indicated that it was a primary GBM. The gain corresponding to the CDKN2A locus is uncharacteristic for this locus in high malignancy grade astrocytomas. If the CDKN2AandCDKN2B are involved in the tumour they may be operating under different mechanisms, perhaps with implications on prognosis. Alternatively it may be that gene(s) other than those usually implicated in astrocytomas in this location are responsible for pathogenesis of this tumour.

6-5-4-4 GBM/S2687m

GBM/S2687 is a recurrent glioblastoma whose original category could not be established. Cytogenetic analyses revealed gains, possibly of EGFR (7p12), PDGFA or PMS2 (7p22) and deletion, presumably of PTEN (-10q). These alterations suggest that GBM/S2687 might have been a primary glioblastoma. However, amplification at the locus of PMS2, which is a mismatch repair (MMR) gene, might imply that the patient was susceptible to other genetic disease such as Turcot's syndrome, for example, and associated malignancies. Indeed, the large deletion at 16p could implicate the TSC2 gene, another gene with established roles in syndromes with astrocytoma association. If this were the case, this tumour would also fit the category of a secondary GBM.

6-5-4-5 GBM/S1575

This tumour gained at regions corresponding to loci of EGFR and CDK4/MDM2, and lost at loci of PTEN. These CNAs would suggest that this tumour is a primary GBM. This is in agreement with the clinical categorization of this tumour. However, this tumour had copy number gains at the locus of PMS1, and deletions at 16p 13.3-13.2 (implicating the TSC2 gene) and 19q12. There were also 4 regions of novel gains and 6 of loss. Most of these were discrete alterations that could point to oncogenes and TSGs with roles in development of this primary GBM.

6-5-4-6 GBM/S3044

GBM/S3044 had accumulated very many CNAs, with at least 13 gain-CNAs and 14 of loss, covering most of the chromosome arms. It was not possible to obtain clinical data on the category for this tumour. However, it was deleted at 17p13 (implicating TP53) and at 1p and 19q, and had gained at loci implicating EGFR and CDK4/MDM2. There were additional gains at loci implicating PMS1 and APC, and losses at loci of TSC1, TSC2 and NF2. Deletions of TP53 with concurrent loss of 1p and 19q would suggest that this tumour was a progressive GBM, presumably one with the potential for a better prognosis. This pattern would suggest that GBM/S3044 could be of the progressive type.

6-5-5 Tumours with CNAs at 4 loci of genes with established roles in development of sporadic astrocytomas

6-5-5-1 GBM/S1595m

Remarkably this tumour had aberrations implicating 4 genes with established roles in pathogenesis of sporadic astrocytomas and 5 other genes with equally well-established roles in syndromes associated with astrocytomas. In addition there were also discrete deletions at 1p and 19q, a combination that has been associated with tumour prognosis both in oligodendrogliomas, mixed oligo-astrocytomas, and other gliomas. The combinations of CNAs, which include +EGFR, +CDK4/MDM2, +APC, -TP53, -1p, -19q, -TSC1/2, -NF1/2 would more likely suggest the possibility of it being a secondary GBM. In fact, clinical data obtained indicated that it was a primary

GBM, which is of interest considering the apparent wide-ranging involvement of loci of genes with roles in hereditary syndromes with astrocytoma association and those that have specifically been associated with sporadic astrocytomas. Another equally interesting observation is the gain corresponding to the locus of RB, which is uncharacteristic for this gene in high malignancy grade astrocytomas. If it is involved in the pathogenesis of this tumour then it may be operating under different mechanisms, perhaps with implications on prognosis. Alternatively it may be that another gene at this locus is responsible for pathogenesis of this tumour.

6-5-5-2 GBM/S1625

GBM/S1625 has the classical combination of alterations, +7 (spanning 7p22 and 7p12) implicating EGFR and PDGFA/PMS2), +12q14-q15 (implicating CDK4/MDM2), and -10q (implicating PTEN), that could place it in the category of a primary GBM. This is in agreement with clinical data on the category of this tumour. However, the probable involvement of PDGFR (or of PMS2) in 7p22 could have also placed this tumour in the category of a secondary GBM. Additional gains at 5q21-q22 (implicating APC) and 7q, which were the only other CHR regions gained, would suggest that other oncogenes with roles in pathogenesis of astrocytomas might be located in these chromosome arms. In addition, there were other regions of copy number loss. Other than the entire loss of 10 (p/q), novel discrete losses were at 2q35-37 in which 5 other tumours lost with only one spanning this locus. The second loss was at 13q33-34 in which 4 other tumours also lost. Interestingly, the same set of tumours (except one; /C160) lost at both loci.

6-5-6 Tumour with 5 CNAs at loci implicating genes with roles in development of sporadic astrocytomas

6-5-6-1 GBM/S2858

GBM/S2858 with its combination of CNAs involving +7p12, +PDGFA/PMS2, +12q14-q15, -CDKN2AandCDKN2B, and -17p13 fits into either category of GBM. However, the clinical data categorizes it as a primary GBM, which raises issues with the probable roles of losses at 9q34, 17q11.2 and 22q12.2 (implicating TSC1, NF1, and NF2), three established astrocytoma susceptibility syndrome genes in the pathogenesis of this primary glioblastoma.

6-6 Deletions at 1p and 19q

Combined deletions at 1p/19q are probably the most relevant prognostic genetic predictors in grade 2 and 3 oligodendrogliomas (van den Bent et al., 2006; Cairncross et al., 2006). The combination is also predictive of chemosensitivity in low-grade astrocytomas (Kujas et al., 2005; Hoang-Xuan et al., 2005) and high-grade oligodendrogliomas (Cairncross et al., 1998). As a result, many studies are aiming to establish whether similar benefits can be derived in patients with high-grade astrocytomas. In this current study, losses at 1p were clustered at 2 regions, respectively centred at 1p34.1-p35.3 (in 5 cases) and at 1p36.1-p36.23 (in 8 cases). This is in agreement with other studies that have reported losses at 1p in astrocytomas ranging from 20-30% (Barbashina et al., 2005). The data could suggest that these regions harbour at least two genes that could be involved in pathogenesis of astrocytomas. Similarly, allelic loss of chromosome 19q is observed frequently in gliomas, and it has been suggested that terminal or interstitial deletions occur predominantly in astrocytomas and glioblastomas whereas tumours with an oligodendroglial component appear to lose the entire long arm of chromosome 19 (von Deimling et al., 1994). Chromosome 19 was one of the most frequently altered in high-grade astrocytomas in this study. Of the 11 tumours with CNAs in the 19q-arm, 4 (~37%) had extensive deletions in the q-arm, demonstrating that extensive losses in the 19q-arm occurs in a sizeable proportion of astrocytomas of high malignancy grade.

Although no genes have yet been confirmed, allelic losses on CHRs 1p and 19q have emerged as potentially important determinants of prognosis and response to therapy in gliomas in general, but in particular oligodendrogliomas (Cairncross et al., 1998; Jaeckle et al., 2006). In the study published by Kujas et al., in 2005, it was shown that in low-grade gliomas, the 1p/19q deletion is more predictive of survival than even the histological subtype (i.e., oligodendroglioma, astrocytoma or oligoastrocytoma). However, several other equally recent studies have reported that the site and extent of deletion, and the grade of the tumour may be important factors (Ngo et al., 2007; Idbaih et al., 2005). The study by Idbaih and colleagues, reporting large-scale genomic analysis by array CGH in 2005, showed 2 clearly distinct patterns of 1p deletion in gliomas. Deletions of the whole of 1p, when associated with whole 19q deletions, were related to typical oligodendroglial morphology and good prognosis. More relevant to the current study Idbaih and co-workers also studied 35 cases of glioblastoma multiforme, of which two had a loss of the whole of 1p, fifteen had a loss of 1p36 and eighteen had no loss on 1p. In these patients the loss of 1p36 was associated with significantly worse prognosis (P=0.01). The molecular mechanisms that underlie the association between 1p/19q loss and chemosensitivity remain unknown because the genes targeted by 1p/19q deletions have not been identified.

Three recent randomized phase-3 trials failed to show a significant benefit of adjuvant chemotherapy in grade 3 oligodendrogliomas and oligoastrocytomas in cases of 1p/19q-deleted tumours and in those with no deletion at these loci. The 1p/19q loss identifies a favourable prognostic sub-group of oligodendroglial tumours, with a median survival longer than 8 years compared to 2 years (Jenkins et al., 2001; European Organization for the Research and Treatment of Cancer 26951) and 2.8 years (Kros et al., 2006; Radiation Therapy Oncology Group 94-02) for tumours without 1p/19q loss, independent of treatment. The progression-free survival after radiotherapy-only in patients harbouring 1p/19q loss tumours is also much better (van den Bent et al., 2006 (EORTC); Cairncross et al., 2006 (RTOG). A very recent review of progress in treatment of gliomas (Idbaih, 2007) confirms that in oligodendrogliomas the detection of 1p/19q deletions has strong implications for prognosis although this has not yet translated into success in targeted treatment.

In the current study of sporadic astrocytomas, 1p deletions in all 9 tumours were discrete, which would be in agreement with findings of Idbaih and co-workers. Most

of our tumours with discrete deletions at 1p36 (n=8) also had deletions of 19q (n=6) of which 4 (50%) involved the entire 19q arm.

The next section considers whether any of the molecular data from the current study show any correlation with survival.

6-7 Survival of patients investigated in this study

Survival data were available for 26 (~80%) out of 32 patients in the study, of which 5 had WHO grade III astrocytomas (figure 6-6) and 21 had glioblastomas (figure 6-7). Ten patients (2 with anaplastic astrocytomas and 8, glioblastomas) died within three months following initial diagnosis and treatment. This would suggest that this group of patients presented with advanced disease at diagnosis or/and had iatrogenic causes of mortality.

The majority of patients in this study were relatively young, mostly in middle age. From table 6-2 it can be seen that the ten patients already mentioned who survived three months or less had a mean age of 51.5 (median age about 49) and the eight who survived between 4 and 11 months have a mean age of 49.75 (median age about 45). The eight who survived twelve months or more were not significantly younger (mean 45.75, median age about 44.

Table 6-2 - Survival data for 26 patients with high malignancy grade astrocytomas

	Tumour ID	Grade	Age/sex	Survival (months)
1.	AA/C545	III	38/M	2
2.	AA/S2614	III	40/F	13
3.	AA/S2706	III	38/F	59
4.	AA/S2721	III	46/M	1
5.	AA/2745	III	31/M	18
6.	GBM/S11	IV	53/M	1
7.	GBM/C160	IV	40/M	19
8.	GBM/C1397	IV	37/M	3
9.	GBM/C1510	IV	55/M	40
10.	GBM/S1575	IV	71/F	2
11.	GBM/S1595	IV	68/F	18
12.	GBM/C1612*	IV	44/M	8
12.	GBM/C1724*	IV	44/M	8
13.	GBM/S1706	IV	47/M	44
14	GBM/C1719	IV	69/F	2
15.	GBM/C1752	IV	44/M	7
16.	GBM/C1760	IV	57/F	2
17.	GBM/S2051	IV	44/M	1
18	GBM/S2093	IV	55/M	11
19.	GBM/S2650	IV	44/M	11
20.	GBM/S2126	IV	64/M	10
21	GBM/C2394	IV	47/M	17
22	GBM/S2409	IV	41/F	5
23	GBM/S2532	IV	50/M	2
24	GBM/C2685	IV	44/M	11
25	GBM/S2687	IV	48/M	3
26	GBM/S2848	IV	46/M	7

AA = anaplastic astrocytoma (WHO grade III), GBM = glioblastoma multiforme (WHO grade IV), S = solid/biopsy & C = cell cultures, respectively representing abbreviations for

the source of DNA, which was used in the CGH experiments in this study. M =male, F = female. Survival data is in months after initial diagnosis *Tumour specimens of one patient

Patient survival is known to depend on several other factors, for example, intracranial site/location of a tumour, extent of surgery, and the mode and dose of radiotherapy administered. Clinical data relating to most of these parameters were not available for this study, which made it difficult to be more specific about their possible role in these early deaths, or generally the overall outcomes. The CGH data from these 10 patients were very variable. But the most frequent chromosomal aberrations involved gains of CHR 7p in 5 patients (50%), CHR 13 in 5 patients (50%), and CHR 7q in 4 patients (40%). Meanwhile, the most commonly deleted regions were at 16p and 19p each in 5 patients (50%), and 1p, 10q, 16q, 17p, 19q, and 22q, each in 4 patients (40%) who died within three months of diagnosis. The rest of this section will divide the patients into those with anaplastic astrocytomas (5 with survival data) and those with GBM (20 patients).

The median post-treatment survival of patients with anaplastic astrocytomas has been reported as 3 - 5 years (Surawicz et al., 1998; Sant et al., 2003). Recent improvements in surgical access and extent of resection, in addition to more accurately targeted radiotherapy, may have resulted in modest improvement in survival of patients diagnosed with glioblastoma multiforme. The median post-treatment survival is currently around 12 months (Brandes et al., 2004; Mitchell et al., 2004). Therefore, it appears that a fair proportion of glioblastoma patients now survive for 1-2 years after diagnosis. Nevertheless many studies and reviews continue to report an overall duration of survival of less than 1 year for glioblastoma. In this study, only one out of 3 patients (33%) with anaplastic astrocytoma, a female aged 38 years, survived for approximately 5 years (see figure 6-6). Two other patients with AAs survived for 13 and 18 months respectively. This was far below the expected median survival for this grade of astrocytoma. Except for the possibility of iatrogenic causes of death, which could have accounted for the death of two patients with AAs who died within three months of diagnosis, the short duration of survival in four of the five patients with AAs would suggest that they might have had tumours with more aggressive molecular characteristics, or that they died of other causes. The CNAs found in these patients are shown in figure 6-6. Indeed AS2745 had 8 discrete gains, which included three that

are located on similar chromosome arms in AS2614 (3p, 61 and 13q) and 9 losses, one of which (16q11.2) is common to both tumours. These regions could harbour genes that modify the biological character of astrocytomas, perhaps conferring an aggressive phenotype. The patient who survived 18 months did have discrete deletion of 1p36 and large (whole) deletion of 19q, but the possible role of these is unknown

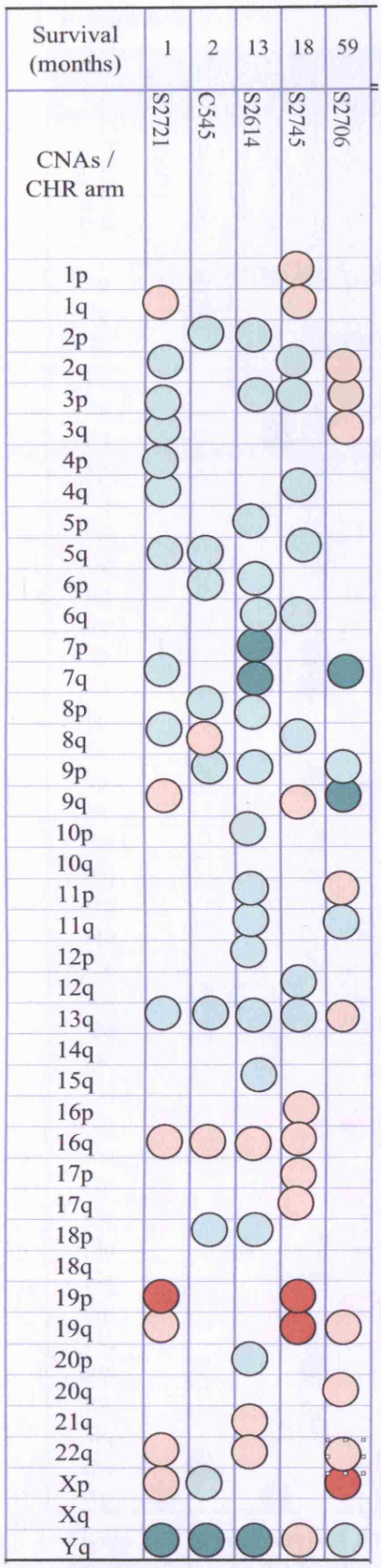


Figure 6.6: Survival of 6 patients with AAs

A summary of chromosome abnormalities with tumours arranged according to duration of survival of the patients (months) following initial diagnosis

Figure 6-7

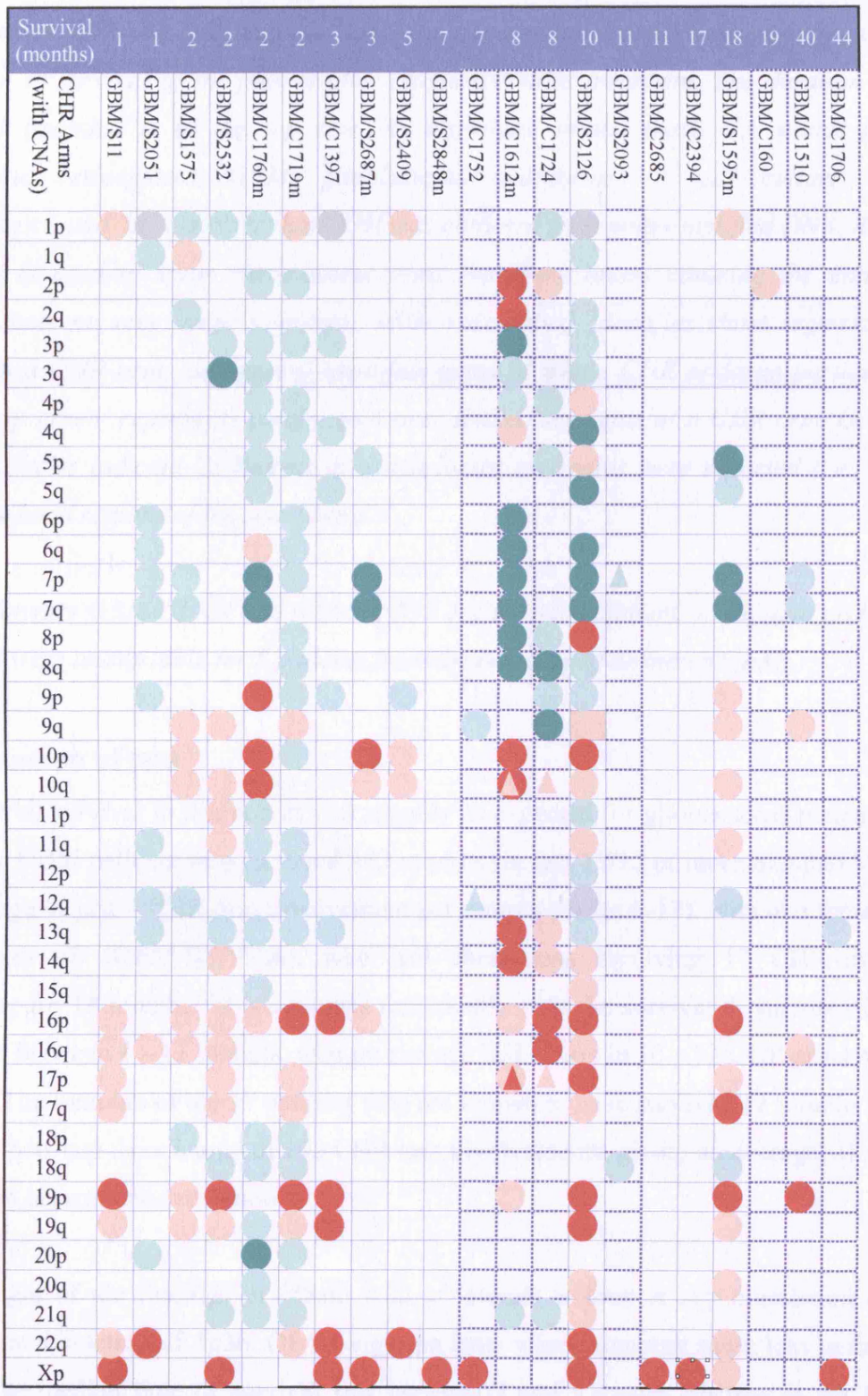


Figure 6-7 shows survival data for 20* patients with GBMs - a summary of chromosomal abnormalities with the tumours arranged according to the duration of survival of the patient after initial diagnosis.

* The tumour GBM/C1724 and GBM/C1612 are the same patient, while data on the survival were unavailable for 5 from the category of unknown GBMs.

Explanation for Figure 6-7– Survival data of 20 patients with GBMs*

A summary of chromosome abnormalities with the tumours arranged according to the duration of survival of the patient after diagnosis/initial treatment. The duration of survival (months) is in the top row. As elsewhere in the thesis AA stands for anaplastic astrocytoma, GBM: glioblastoma multiforme, C: cell culture, S: solid/biopsy, and 'm' indicates that CGH was obtained from mda-amplified DNA. Red shading is used to show chromosome arms that bore losses covering the entire chromosome or very large segments, while rose (pink) identifies short segmental losses in a CHR arm, 'sea-green' identifies gains of whole CHR or large portions, and 'light green' represents patchy gain over limited segments of a CHR arm. Grey shaded circles indicate CHR-arms in which losses and gains were reported (i.e., in different band regions of the same arm)

** The tumours GBM/C1724 and GBM/C1612 are the same patient, while data on the survival were unavailable for 5 patients from the category of unknown GBMs.*

Continuation of text...

The overall survival in this cohort was roughly as expected for glioblastoma patients. The five GBM patients who survived >12 months (in fact 17/12 or more months) had an average of just 4 CHR-arm involvement per tumour (range 0-17), with one female aged ages 68 (GBM/S1595/m), who had aberrations involving 17 CHR-arms surviving for 18 months. This contrasts remarkably with the survival 8 patients who survived between 4 – 11 months, who on average had CNAs in 10 – 12 CHR arms per person. The tumours of the 17 patients who are known to have survived 11 months or less had between them a total of 192 CHR arm involvements, giving an average, of 11 CHR arm aberrations per tumour.

In the light of the findings of Idbaih it is of interest to look at any correlation of survival and deletion of 1p36. Of the eight patients whose tumours show loss in this region the median time of survival was between 3 and 5 month whereas in the 13 patients without this specific change the median survival was 8 months. However five very short-term survivors (<4 months) had other changes in 1p. The median survival for 10 GBM patients with no changes on 1p was 11months. These data are consistent

with the findings of Idbaih and coworkers although the numbers are too small to draw firm conclusions.

6-7-3 Long-term glioblastoma survivors

Two (10%) out of 20 patients with glioblastoma in this series survived for 3 or more years. In the context of glioblastoma multiforme a patient is considered to be in the category of long-term survivors if the survival outcome is at least three years (Scott et al., 1999; Burton et al., 2002). Although our series is quite small in comparison, the finding of 3-year survival of ~10% is somewhat in agreement with that of Sant and colleagues (2003), who investigated management outcomes of 6,513 'adult' glioblastoma multiforme patients in a recent EUROCARE study, except that the higher proportion of survivors (11%; range 2 – 11%) in their series was attributed to patients aged 15 – 18 years – by far a much younger age group of patients than those in this series. This data indicates a higher proportion of glioblastoma survivors than currently thought possible (Senger et al., 2003; McLendon and Halperin, 2003). This would either validate the view that there has been some improvement in current management practices for glioblastoma, or/and that a fair proportion of glioblastoma subgroups may possess molecular markers responsible for long-term survival.

On the basis of molecular cytogenetic alterations observed by metaphase CGH (figures 6-7) a general observation that is striking from these two long-term survivors (LTSs) is the relatively small number of copy number alterations that were reported in their tumours.

From these CGH results, the two LTSs shared no common aberration. GBM/C1510 had gains at several minimum common region of overlap (MCROs) on CHR 7 (p & q), and losses at 9q34, proximal 16q, and 19p, while GBM/C1706 had gains at 13q21 and the entire X-CHR. Neither tumour showed a loss at 1p36

6-8 DNA Copy Number Aberrations implicating genes of syndromes with astrocytoma associations

As already mentioned, DNA copy number aberrations implicating several loci of well known syndrome-genes, such as those for tuberous sclerosis and neurofibromatosis, were found at a frequency similar to, and in some cases higher than at well known loci like those of PTEN, TP53, CDKN2AandCDKN2B and RB1. The latter are considered as the major contributors to pathogenesis of sporadic astrocytomas while the former group have established roles in astrocytomas associated with hereditary /familial syndromes. Hereditary astrocytomas are believed to account for ~5% of astrocytomas, the majority being pilocytic, benign, WHO grade 1, astrocytomas. Although generally well circumscribed and progress slowly, NF1-associated pilocytic astrocytomas (NF1-PA) occasionally behave as aggressive tumours (Tada et al., 2003), suggesting that certain aberrations associated with the NF1 gene are capable of causing malignant transformation. The low expression of NF2 gene in hereditary astrocytomas (Weber et al., 1996; Watkins et al., 1996) and of the TSC1/2 genes (Weber et al., 2007), implicates these genes in pathogenesis of sporadic astrocytomas. Although the evidence in each case directly relates to hereditary astrocytomas, it would seem to provide indirect evidence for sporadic astrocytomas that have LOH at these loci. Further evidence pointing to a possible role of these genes in sporadic astrocytomas is implied by:

1. Occurrence of glioblastomas in association with congenital syndromes, for example, the NF1/2 (Lee et al., 2006; Kruger et al., 2007) and TSC1/2 (Malmgren et al., 1984; Al-Saleem et al., 1998; Senatus et al., 2005)
2. Involvement in tumours of other glial cell types e.g., ependymomas (Zheng et al., 2000)
3. And, in addition they have been implicated in pathogenesis of sporadic tumours of other cell-types and organs; for example, the NF1/2 genes in colorectal cancer (Rustgi et al., 1994/1995; Cacev et al., 2005), and the TSC1/2 genes in cancers of the bladder and other sites (Knowles et al., 2003; Adachi et al., 2003; Rosner et al., 2006).

It seems of interest that past studies failed to establish a clear association between these genes and sporadic astrocytomas. Indeed, despite the good overall agreement between metaphase and array CGH data in this study, there were notable discrepancies in regions harbouring the TSC1/2 in 3 tumours (showing alterations in the metaphases) among the small group investigated by these two procedures. This renders the data somewhat inconclusive on the possible role of the TSC1/2 genes in these tumours and the study in general. The question of the adequacy of either/or both

CGH procedures (metaphase and 1MB arrays) for investigating aberrations in the chromosome regions that harbour these genes has been raised by several other observations. A genomic array similar to the ones used in this study also failed to detect established deletions of the TSC2 gene in 2 samples (Michaelet et al., 1997; Ekong et al., 2004). Among other possible explanations, the failure to validate previously established deletion, by Ekong and co-workers, one reason would be that the genomic arrays were not sensitive enough to detect the aberrations in the TSC2 region possibly because of polymorphism in the repetitive sequences not fully suppressed by COT1 DNA (for example, see Radon et al., 2006). This problem may be overcome eventually by using arrays containing only genuinely single copy sequences.

Therefore, while the CGH methodologies measure the relative DNA copy number of one test genome compared to a reference, they overlook its absolute DNA ploidy state and the distribution of common repeats across the genome, which can impair data interpretation (Carter et al., 2002; Mantripragada et al., 2004; Fiegler et al., 2007), and are also insensitive to balanced rearrangements such as inversions and reciprocal translocations. Although segmental duplications and structural polymorphism are common to many chromosomes, in general chromosomes 9, 16, 17 and 22, which harbour the genes for NF1/2 and TSC1/2 are known to have relatively higher percentages of repetitive sequences and segmental duplications, as well as being structurally polymorphic within the substantive euchromatic regions (Humphray et al., 2004; Martin et al., 2004; Mantripragada et al., 2003/2004; Zody et al., 2006). It would therefore require the use of more specific and elaborate methodologies to search for aberrations at the loci of these genes. On the other hand, it is possible that some other genes, located in the vicinity of the well-known hereditary-astrocytoma

associated genes, are the ones that are altered and responsible for sporadic astrocytomas. However, no alternative candidate TSGs have so far been identified, and the search continues both in glial tumours and tumours of other sites that have been found to show frequent LOH in the regions of these genes (for example, Ho-Keung et al., 1998; Knowles et al., 2003; Suarez-Merino et al., 2005; de Stahl et al., 2005).

6-9 So, why is it that genetic studies of sporadic astrocytomas have so far failed to find evidence firmly implicating some well-known syndrome-genes, in particular the NF1/2 and TSC1/2?

This concern is also at the centre of this current study; the two key issues arising from it are: 1) whether genes implicated in established syndromes (with astrocytoma association) contribute to development of sporadic astrocytomas, and 2) why, in spite of frequent findings of LOH (and in some cases, apparently reduced gene expression), that studies of sporadic astrocytomas have so far failed completely to find evidence firmly implicating some well known syndrome genes in the pathogenesis of sporadic astrocytomas.

There may be several possible explanations. LOH does undoubtedly occur in some sporadic astrocytomas but may be quite uncommon and may be overestimated by CGH approach. This problem should be solved by better arrays, including SNP arrays, and by recognizing the essential need to have normal DNA from the patient – a need emphasized by recent recognition of the extent of normal copy number variation of large segments (Redon et al., 2006). However, if the LOH is genuine and important, another explanation is haploinsufficiency of genes implicated.

6-9-1 The role of haploinsufficiency

Based on the evidence of LOH it may be that these TSGs can contribute to astrocytomas via haploinsufficiency rather than the canonical two-hit hypothesis. Several investigators now believe that this may be the case (for example, Gutman et al., 1999; Paige AJW, 2003; Barbashina et al., 2005). However the associations of haploinsufficiency and tumour occurrence requires cautious interpretation due to strain-specific variations (Hawes et al., 2007).

6-10 An Illustration of Probable Molecular Pathways of some GBM Observed in this Study.

It has been generally accepted that glioblastomas are distinguishable from anaplastic astrocytoma by, among other possibilities, primarily, deletion of 10q (Somerville et al., 1998; Fults et al., 1998; Kleihues and Cavenee, 2000; Furuta et al., 2004), which is thought to implicate the gene for phosphatase and tensin homolog (PTEN) at 10q23.3. Based just on this premise our panel of glioblastomas can be categorized into at least three subgroups. 1) Those with deletions of 10q spanning the locus for PTEN - possibly the category that conforms to the current definition, 2) those with deletions of 10q but in which the PTEN locus is apparently spared, and 3) histologically confirmed glioblastomas that did not show copy number alterations anywhere in chromosome 10. As far as the status of the PTEN locus, as determined by metaphase CGH is concerned, the latter two categories appear to be at variance with the prevailing molecular definition of a glioblastoma. This would suggest that alternative molecular endpoints exist for some categories of glioblastoma multiforme. However it is important to keep in mind that deletions of the PTEN gene may have occurred but could be too small for detection using the method of metaphase CGH. Indeed array CGH of GBM/C1724 found deletions of PTEN which where not revealed by the metaphase CGH experiment. On balance, the finding of a region of loss or gain in a specific clone for which a prior hypothesis exists is therefore a firmer result than the failure to find one (Sue Povey, personal communication).

This study observed 2 regions that were at the centre of most deletions, or minimum common region of deletions (MCRD) in the 10p-arm. These were located respectively at 10p11.21 and 10p11.22-p11.23. Similarly there were three MCRD in the 10q-arm centred at 10q21.2-q21.3, 10q23.1-q23.3 (encompassing the locus for PTEN), and 10q25.1-q26.2. These 5 most commonly deleted regions on chromosome 10 might harbour TSGs that may constitute different molecular pathways (perhaps endpoints) for some categories of glioblastoma multiforme (for details, please refer back to the section on CNAs of CHR 10 in Results Chapter 3).

Similarly, inactivation of TP53 is considered a key factor in early transformation of astrocytomas along the pathway to secondary GBMs (Jin et al., 2000; Fulci et al.,

2002; Wang et al., 2004). TP53 is highly homologous to TP73 and the two genes are considered to be functionally similar (Benard et al., 2003). MDM2 is normally bound to TP53, rendering both of them inactive (Issaeva et al., 2004; Arva et al., 2005). Amplification of MDM2, which results in free, 'unbound' MDM2 is considered to be a primary molecular mechanism for bypassing tumour suppressor functions of TP53 (Carneo et al., 2000; Yu et al., 2003). Thus, the three genes, TP53, TP73 and MDM2, may have critical roles in early tumour development in some categories of astrocytic tumours. Data from this study appear to suggest that there may be alternative mechanisms for bypassing p53 tumour suppressor functions in glioblastomas. A comparison of the pattern of CNAs in loci implicating TP53, TP73 and MDM2 found a total of 14 tumours with CNAs implicating one or more of these loci (Table 6-3), with another 18 revealing no CNAs in any of the above loci (see figure 6-3 for a detailed plot of CNAs).

6-9-1 Table 6-3

Gene/Locus	AA	Glioblastomas (GBMs)												
	S2745	S11	C1397	S1575	S1595/m	S1625	C1719/m	S1926	S2051	S2126	S2409	S2532	S2858	S3044
TP53 [17p13.1]	○	○	○		○		○			○		○		○
TP73 [1p36.3]	○	○			○			○	○	○	○			○
MDM2 [12q14.3-q15]	●			●	●	●			●	●			●	●

Table 6-3 shows CNAs in loci of 3 genes: TP53 and two others that may have roles in astrocytoma oncogenesis. An empty box signifies that no CNAs were observed in a corresponding gene locus, circles filled with either "tan" or "green" colours (respectively representing loss and gain) identify loci implicated in copy number aberrations. The CHR region harbouring TP53 and TP73 were deleted, while that of MDM2 was gained.

Gains of the region containing the locus for MDM2 implicated it in the development of 3 out of 14 cases (~21%) in which the above three genes are implicated. On the other hand the CHR region containing the TP53 locus was deleted alone in 3 cases (21%) and that of TP73 in 2 cases (14%). Probable cooperation of regions of all three genes, accounting for the majority in which more than one of three genes was implicated, was observed in 4 (28.6%) cases. Cooperation between regions harbouring TP73 and MDM2 occurred in one instance (7%), while loci corresponding to TP53 and TP73 were jointly deleted in 1 case (7%). From previous work (Biernat et al., 1997; Ghimenti et al., 2003) it has been suggested that deletion of TP53 and amplification of MDM2 are alternative mechanisms rarely found in the same tumours. In this series there are 4 cases of gain of the region harbouring the MDM2 without apparent loss of TP53, and similarly 4 cases of loss of TP53 without obvious gain of MDM2. There are four tumours where there is apparent loss of TP53 and gain of MDM2. In these 4 cases, and indeed in four others, there is also a loss of regions containing TP73. This might suggest involvement of different genes. The respective regions implicated here harbour multiple genes of interest, in particular the region to which the MDM2 gene is mapped, at 12q13-q15 (Fischer et al., 1996; Reifenberger et al., 1996; Nakahara et al., 2004). On the other hand, the same genes may be involved, with some acting via different mechanism, which could have implications on prognosis. However, irrespective of the operational mechanisms, or the specific combinations of genes that might be implicated, this possibility further implies existence of subgroups within this category of astrocytic tumours.

Thus, considering the probable involvement of these three genes that are believed to have critical early roles in transforming a subcategory of tumours of astrocytic origin, this study appears to identify at least 7 subgroups of high-grade astrocytic tumours six of which are within this subcategory alone. It is likely that epigenetic mechanisms may account for some cases, which would further point to additional subgroups. That aside, this study suggests that some astrocytomas do not have gross structural alterations involving the chromosome regions harbouring loci of the genes for TP53, TP73, and MDM2, which in this study amounts to 21 (~65%) tumours. It is possible of course that some of the tumours could have small deletions or amplifications, or indeed point mutations in these regions. However since major structural changes are an extremely common basis for the evolution of tumours, it is certainly possible that

these tumours have arisen by different mechanisms. This suggests that other initiating genes may be responsible for tumour development in this latter group, which would also imply additional subcategories. Eleven of the 21 tumours that do not appear to have gross alterations of TP53, TP73 and MDM2, had copy number aberrations suggesting deletions at several other gene loci, of which at least 19 may harbour tumour suppressor genes that could have contributed to tumour initiation in this set of astrocytomas. Please refer to figure 6-3 for details.

6-11 CONCLUSIONS

There are striking differences between primary and secondary glioblastomas with respect to clinical manifestations as well as genetic aberrations (Ohgaki and Kleihues, 2007). For example, there are differences in age distribution of patients, with the mean age of primary GBM patients being ~60 years, whereas patients with secondary GBM are on average ~45 years (Ohgaki et al., 2004; Ohgaki & Kleihues, 2005). There is also gender bias; primary GBMs develop more frequently in men (M:F ration, 1:33), whereas secondary GBMs are more frequent in females (M:F ratio, 0:65 (Ohgaki et al., 2004)). In terms of survival, the median survival of secondary GBM patients (7.8 months) is marginally better than of patients with primary GBMs (4.7 months) (Ohgaki & Kleihues (2007). However, this difference is considered largely due to the younger age of patients with secondary GBMs (Kleihues et al., 2004). Other than age, which is a consistent predictive factor of longer survival of GBM patients, multivariate analyses have so far showed no significant differences in survival of patients with primary and secondary glioblastomas (Ohgaki et al., 2004; Ohgaki & Kleihues, 2005). This seems to emphasize a need for a better understanding of the clinical manifestations and the associated molecular pathogeneses, for the respective sub groups of tumours, so as to design effective targeted molecular therapies. The work described in this thesis has been one small step in this complex process. The results have shown considerable agreement with some established previous findings for example gains on chromosomes 7 and 12 and losses on chromosome 10. There was also some agreement with less well established previous findings for example fewer chromosome aberrations in tumours from patients who survived longer and some suggestion of a worse prognosis for tumours showing loss in the 1p36 region

As noted previously, one of several additional observations from this study is that loci of almost all the genes currently implicated in familial/astrocytoma susceptibility syndromes, such as the genes for tuberous sclerosis and neurofibromatosis, are predominantly implicated in sporadic astrocytomas investigated in this study. This would suggest that these genes (or others thereabout) despite several of them not featuring in current models, could have contributed to pathogenesis of these sporadic astrocytomas. Regrettably, because of the limited access to micro-arrays these

metaphase CGH data could not be adequately compared with the microarray in some of the regions of genes that we would be interested to investigate further, i.e., at 9q34 (TSC1), 16p13 (TSC2), 17q11.2 (NF1) and 22q12 (NF2). These genes (and regions) have been frequently investigated in the past and no definite role in sporadic astrocytomas has been found (refs, as indicated previously). Indeed, this study found a discrepancy involving the TSC2 region in one tumour; the convincing loss of 16p13 in 1724 that was not confirmed on microarray. This would appear to support the earlier findings of other investigators mentioned above. However, equally, the discrepancy in this study involving the TSC2 region would appear to cast some doubt on the sensitivity of microarrays in investigation of this region. Ekong et al., in 2004 published a paper in which a microarray failed to detect established deletions of 69 kb and 136 kb (Michalet et al., 1997) in two samples they examined. Results obtained for the cosmids in the TSC2 region were inconsistent and indicated either a deletion for all the clones including those known to be undeleted, or normal ratios for all clones including the deleted ones. The spurious results found in this region may be attributed to the fact that it is known to have a high density of repeat elements and duplications (Sampson et al., 1997; Bailey et al., 2002). Furthermore, labelling artifacts are known to cause wide variation of fluorescence ratio in heterochromatic pericentromeric regions (1, 9, 16) and GC-rich regions for example on chromosomes 1pter, 16, 19 and 22. These limitations of the CGH procedures could account for discrepancies observed between metaphase and microarray CGH in some of the chromosome regions, for example at 9q34 (in 1510), 16p13 (in 1612 and 1724), and 19p13 (in 1510 and 1612) that were highlighted by this study. In spite of these difficulties of interpretation the data in this study which suggest loss in the regions implicated in hereditary astrocytoma syndromes are too strong to be dismissed.

An additional issue that arises from these data is the very diverse nature of aberrations revealed by metaphase CGH in a relatively small sample of high-grade sporadic astrocytomas (Table 6-1; Figure 6-3). While a number of these aberrations are typical of changes that have long been associated with various grades of astrocytomas, several others are largely unreported in the literature on astrocytomas. This could raise issues concerning the cellular and molecular basis of glioblastomas. The cell of origin of secondary glioblastomas may be somewhat straightforward, as it would be inferred from the histological diagnosis of a preceding, lower grade tumour that

would have progressed or recurred. However, with regards to primary glioblastomas, a rapidly developing subgroup of tumours, which are believed to constitute approximately 90% of glioblastomas, the anaplastic histology obscures the normal morphology, creating uncertainties with defining the cellular origins of such tumours. While there is no disputing the fact that astrocytes could contribute to a significant proportion of primary glioblastomas, it seems that the assumption of an astrocyte as the inevitable cell of origin may be in some doubt. This view is illustrated by the case of glioblastomas arising from oligodendrocytes. Moreover, reliance on GFAP immunohistochemistry for resolving uncertainty with the diagnosis following anaplastic histology seems unspecific. This examination appears not to be entirely specific to astrocytes (Walz and Lang, 1998; Wittowski W et al., 1998), and is also uninformative for subgroups of astrocytes and/or glioblastomas (Reifenberger et al., 1987; Walz Wolfgang, 2000; Chronwall et al., 2000; Riemenschneider et al., 2004).

Secondary glioblastomas arising from oligodendrocytes might be expected to retain some characteristic histological features suggestive of the cell of origin (Ghimenti et al., 2003). However, the fact that oligodendrocytes give rise to secondary glioblastomas strongly suggests that this cell type could give rise to primary glioblastomas, perhaps with little if any distinguishing histological features. If the histologies of primary glioblastomas that arise from astrocytes and oligodendrocytes are similar, then it is probable that other glial cell types can give rise to primary glioblastomas with apparently similar histological features. A considerable fraction of gliomas show ambiguous histological features that make their classification into oligodendroglial or astrocytic difficult, and immunohistochemical or molecular markers for unequivocally distinguishing glioblastomas that have arisen from oligodendrogliomas and astrocytomas are not available (Riemenschneider et al., 2004). Even more interesting is the observation that some glioblastomas display neoplastic neuronal elements (Fung et al., 2004; Galli et al., 2004). All these add to the implication that other neuroepithelial cells may constitute significant parts of the anaplastic, neoplastic histology especially of primary glioblastomas. Further still, some glioblastomas have neoplastic mesenchymal features in them (Reis et al., 2000; Bertrand et al., 2002; Alatakis et al., 2005; Orimo A and Weinberg R, 2006; Birnbaum et al., 2007), which suggest that cellular elements traditionally considered extrinsic to the neuroepithelial domain could be involved in glioma pathogenesis. It

seems therefore probable that other neuroepithelial cell types such as ependymal cells and cells destined to form neuronal structures, and even other non-neuroepithelial cell types (e.g., mesenchymal /mesodermal derivatives) might have the potential to give rise to some categories of primary glioblastomas through mesenchymal-epithelial transitions (Orimo A, & Weinberg R, 2005).

Additional support is inferred from profiles of pilocytic astrocytomas. Within the established category of astrocytes, the genetic profiles of pilocytic astrocytomas (WHO Grade 1) are clearly different from those of diffuse infiltrating astrocytomas (WHO Grades II – IV). However, it has been observed that a small proportion of pilocytic astrocytomas progress to glioblastomas (von Deimling et al., 1993; Figueiredo et al., 2003; Luyken et al., 2003; Lubansu et al., 2004). This important observation suggests that cells with genetic profiles typically associated with pilocytic growths have the potential to develop into glioblastomas. Primary glioblastomas are by far the most prevalent of 'astrocytic' CNS tumours. They develop rapidly, most patients typically presenting within ~3 months of onset of symptoms, and with highly anaplastic tumour histology. What proportion of this rapidly developing category of astrocytic tumours might arise from the diverse and varied cell-types mentioned above cannot be precisely ascertained at the moment. While it is true that the changes which influence origin or progression of the tumour presumably arise as random events which confer some proliferative advantage and thus are selected and may come to dominate the tumour, it is nevertheless likely that differences in the original molecular backgrounds of cells from which astrocytomas develop could account for some diversity in molecular profiles that characterise astrocytomas.

6-12 FUTURE DIRECTIONS

This study has produced a broad–brush picture of a large number of tumours. The laboratory procedures that were used allow for genome-wide detection of aberrations in the range of 1-20 Mb. This leaves a diagnostic gap for any alterations in the range of 1-900 Kb. There is also the possibility that some genes could have been affected by point mutation, epigenetic silencing and balanced rearrangements, all of which would have also escaped detection by these methods. As explained previously, both methods are limited in their scope to detect aberrations in regions with high prevalence of repetitive DNA sequences (for example, the long interspersed nuclear elements (LINE) and short interspersed nuclear elements (SINE). In order to further characterize these tumours, it would now require use of more precise tools.

Overall, the study has identified at least 83 regions of recurrent copy number alterations, each shared by a significant number (minimum 3) from a panel of 26 informative high-grade astrocytomas (see in the appendix). Thirty-eight of these are regions of loss, while 45 were of recurrent gains. The low resolution of the techniques used, together with the large number of genes located within these regions, make it difficult to identify candidate genes. First it would be necessary to investigate the remaining tumours using genomic arrays, as was the case for the small number in the current study, allowing identification of the genes implicated in regions identified. Indeed ideally all tumours would be studied again with one of the more recent genomic chips, either a tiling path array or a SNP chip, allowing a more detailed evaluation of LOH. Particularly interesting would be those samples, which have few but definite chromosome aberrations. For the difficult chromosomal regions already discussed, customised arrays (for example exon-specific) might be useful. Quantitative Fluorescent (QF) 5-plex PCR (QF-PCR) of DNA using microsatellite markers might also be helpful for some of these regions.

Having established gene dosage as far as possible there would then be several ways to proceed from this point. As large-scale sequencing becomes cheaper there would certainly be some need for this. Perhaps more immediately, gene expression profiling, for example, using cDNA and appropriate arrays (Pollack et al., 2002; Ruano et al., 2006) would allow the identification of overexpressed (target) genes whose aberrant

expression could suggest involvement in the pathogenesis of the tumours. Additional methods would include quantitative real-time PCR of any candidate genes e.g., those with established roles in sporadic astrocytomas such as EGFR, PDGFA, CDKN2A/2B, PTEN, RB1, TP53, genes with roles in hereditary syndromes, i.e., NF1/2, TSC1/2, and any other genes of interest identified from genomic array CGH data. This would help to answer the question of whether there are other pathways to oncogenesis, which do not include loss of any of these genes, or whether small deletions have been missed. This could also help to explain the apparent low frequency of loss on 9p, 13q, and 17p in this study in spite of previous findings of loss of CDKN2A, RB1, and TP53, in astrocytomas.

The issue of the genes with established roles in hereditary syndromes with astrocytoma association, but which have not been implicated in sporadic astrocytomas, has been of particular interest in this study. It is possible that these genes are not mutated in sporadic astrocytomas as apparently shown by numerous past studies. However significantly reduced gene expression in the absence of demonstrable mutations of the genes might support the hypothesis that they could contribute to sporadic astrocytomas via haploinsufficiency. Furthermore, studies of these regions by the microarrays described above might help to identify alternative candidate TSGs in these frequently altered regions.

In addition to these general possibilities some particular tumours are of interest.
The tumour GBM/C1724 has already been further investigated by microarray CGH and M-FISH, and several findings here would be worth pursuing. There are three areas that would warrant further investigation. The first, is the dramatic gain of material in 4p, shown both in the original (GBM/C1612) and in the recurrent tumour CGH, which microarray has shown to represent a region of ~31Mb. Secondly, array appeared to show several translocation breakpoints in CHRs 1, 8 and 11, while the MFISH of the same tumour revealed numerous translocations, with 4 of them (der(8)t(8;22), der(8)t(8;12), der(9)t(9;11) and der(X)t(16;X)t(17;X)) recurring with a higher frequently.

Chromosome 1 had 3 possible translocation breakpoints, at 1_36.32 flanked by RP4-731G4 and RP11-204L3, and the other, respectively, at 1_150.75 – 1_152.88 (flanked

by RP11-422P24 and RP11-172I6), and 1_160.96 – 1_164.74 (flanked by RP11-541J2 and RP4-702J19). Other possible breakpoints (details in the results chapter) were on 8q and 11p. These (and similar ones in other tumours) would need to be characterized further in the hope of identify possible fusion genes. However, the overwhelmingly large number and diversity of chromosomes in each cell of 1724 may indicate genomic instability. Finally for this particular tumour, it would also be interesting to confirm and define regions where there is apparent loss of a single clone, for example on distal 6q, and alterations in a few scattered outliers in several of the tumours investigated by array (detailed in appendix 4).

Two of the patients with Grade IV malignancy at diagnosis survived for more than three years, and their tumours were also investigated by microarrays. Although one of them showed little (or no DNA) in the sample, these tumours would also be worth further study. Finally, in any study designed in future it would be a great advantage to have more access to clinical information and histology. Microdissection of the tumours would be essential and a parallel DNA sample (probably blood) from each patient would be highly desirable.

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Appendix 1

List of CNAs reported in all 33 analyses of 32 tumours in this study

	Metaphase CGH data		
	Tumour ID	Gains	Losses
ANAPLASTIC ASTROCYTOMAS	S24	None	9q34; 19p13.3-13.2; 22q12-q13
	S2614	2p25; 3p26-21; 5p15.3; **6p25-22**; 6q26-27; 7p22-11.1; 7q21; 7q22-36; 8p23; 9p24; 10p15; 11p15; 11q23-24; 12p13-12; 13q33-34; 15q26; 18p11.3; 20p13	16p11.1-q12; 21p11.1-q12; 22q11.1
	S2706	7q11.2-36; 9p12-q11; 9q21-34; 11q23-24	2q37; 3p21-14; 3q26.3; 11p15; 13q13-14; 19q11-13.3; 20q13.2; 22q13; Xp22.3-q12
	S2721	2q21-q23; 2q31-q32; 3p26-p25; 3q13.1; 4p14; 4q21-q28; 4q31.2-q31.3; 5q14-q21; 7q31; 8q22-q23; 13q21-q22	1q11-q12; 9q11-q13; 15q11.2; 16p11.1-q12.2; 19p13.3-q13.2; 22q13; Xp22.3-p22.2; Xq11-q12
	S2745	2q21-q24; 2q31-q32; 3p12; 4q13-q21; 4q22-q27; 5q14-q23; 6q12; 6q15-q16; 6q21-q22; 8q22; 12q15-q21; 13q21-q31	1p36.3-p36.2; 1q12; 9q11-q13; 16p13.3-p13.2; 16p11.1-q12.1; 17p13; 17p11.1-q11.2; 17q24; 19p13.3-q13.4
	C545	2p25; 5q11.1-q11.2; 6p25; 8p23; 9p24; 13q21; 18p11.3; Xp22.3	8q24.1; 16q23; 22q11.2-q13

Appendix 1

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		Metaphase CGH data	
UNKNOWN GLIOBLASTOMAS	S11	None	1p36.3-p36.1; 16p13.3-p13.2; **16q11.1-q11.2**; 16q24; 17p13; 17q24-q25; 19p13.3-p12; 19q13.3-q13.2; 22q13; Xp22.3-q28
	GBM/S3044	1p33-p13, 1q24-q31, 2q22-q24, 2q31-q32, 3q24-q25, 4p15.1-p14; 4q13-q32; 5q13; 5q14-q23; **6q11**-q22; 7p21-p12; 7q21; 7q22-q31; 11q14; 12q15-q21; 12q24.1-q24.3; **18q11.2**-q12	1p36.3-p33; 2q37; **9q12**, 9q34; 10q25-q26; 13q34; 14q31-q32; **15q11.2**; **16q11.2**; 17p13-p12; 17q24-q25; 19p13.2-q13.3; 20q13.2; **22q11.2**-q13; Xp22.3-q21; Xq21-q28
	GBM/S2848/mda*	Xp22.3-q28	None
	GBM/C1397	1p22; 3p12-**p11**; 4q22-q24; 4q26-q27; 5q14-q22; 9p24; 13q21-q31	1p36.2-p32; 19p13.3-q13.42; **22p13**-q13; Xp22.2-q27
	GBM/1719/mda	**2p11.2-q11.2**; 2p25-p24; 3p12-**p11**; 3p26-p25; 3q28; 4p16; **4q11**-q12; 4q34-q35; 5p15.3; 6p25; 6q22; 8p23; 8q24.2-q24.3; 9p24-p23, 10p15; 11p15; 11q24; 12p13; 12q24.3; 13q34; 18p11.3; 18q22-q23; 20p13; 21q22	1p35-p34.3; 9q33-q34; 16p13.2-**p11.2**; 17p13- q24, 19p13.3-p12; 19q12-q13.3; **22q11.2**-q13; Xp22.1-p21
	GBM/S1575	1p31-p22; 2q21-q24; 2q37; **7p21-q11.2**; 7q21-q32; 12q13-q21; 18p11.3	1q12; 9q11-q13; 10p11.2-q11.2; 10q21; 10q23; 10q26; 13q11; 15q11.2; 16p13.3-p13.2; 16q11.1-q11.2
1° GBM	GBM/C2394	Xp22.2-q27	None
	GBM/C2685	None	15q11.2-q12

*, Tumours that had <3 CNAs (copy number alterations) in the autosomes (n=5).

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		Metaphase CGH data	
	Tumour ID	Gains	Losses
PRIMARY GLIOBLASTOMAS	S1625	1p11-q11; 7p22-q36; **12q13-q15**	2q35-q37; 10p15-q26; 13q33-q34
	S2051	1p22; 6p25; 6q22; 7p21-p14; 7q21; 7q22-q32; 9p24; 11q14; 12q15-q21; 13q22-q21; 20p12-p11.2; 21q11.1	1p36.3-p33; 1q12; 16q11.1-q11.2; 21q11-q12; 22q11-q13
	S2126	1p22-p21; 1q31; 2q23-q21; 3p12; 3q11.1-q13; 4q13-q28; 4q31-q32; 5q12-q23; 5q31-q32; 6q11-q23; 7p21-p11.1; 7q11.2-q32; 8q22; 9p21; 11p14-p12; 11q14-q22; 12p11.2-p11.1; 12q14-q21; 13q21-q22	1p36.3-p31; 1q12; 4p16; 5p15.3; 8p23-p12; 9q11-q12; 9q34; 10p15-q11.2; 10q21; 10q24-q25; 11q12; 12q24.1-q24.3; 14q31-q32; 15q11-q13; 16p13.3-q11.2; 16q23-q24; 17p13-p11.1; 17q21; 17q23-q25; 19p13.3-q13.43; 20q12-q13.2; 21q22; 22p13-q13; Xp22.3--p21; Xp11.4-q21; Xq21; Xq22-q28
	S2409	9p23-p21	1p36.3; 10p15; 10q26.2; 14q31-q32; 22q11-q13
	S2532	1p31-p13; 3p12; 3q21-q27; 13q14-q31; 18q11.2-q22, 21q11.2	9q34; 10p11.2-q11.2; 10q25-q26; 11p15; 11p11.1-q13; 14q31-q32; 16p13.3-p13.1; 16p11.1-q11.2; 16q24; 17p13-p12; 17q24-q25; 19p13.3-q11; 19q13.11-q13.41; Xp22.3-q28
	S2858	1p31; 4q26-q31.1; 7p22-q36; 12q14-q15; 20p13-q12	5q35; 9p24-q12; 10p15-q26; 22q11.2-q13; Xp22.3-q28
	S1595/mda	5p15.3-p15.2; 5p15.1-p13; 5q14-q23; 6q12; 7p21-q11.2; 7q22-q36; 12p11.1-q12; 12q14-q21; 18q26	1p36.2-p33; 9p21-p12; 9q34; 10q24-q26; 11q12-q13; 16p13.3-p11.1; 17p13-p11.2; 17p11.1-q21; 17q22-q25; 19p13.3-p11.1; 19q13.2-q13.3; 20q11.2-q13.1; 22q11.1-q13; Xp22.1-p22.3; Xp11.2-q11; Xq26
	S1926/mda	1q43-q44; 4q34; 5q35; 7p15-p11.2; 7p22-p21; 7q21; 11q23-q24; 12p13; 15q26; 16p11.1-q12.1; 17p13; **20p13-p12**	**1p12-q21**; **9q11-q21**; **16p11.1-q12.2**; Xp21; Xp11.2-q2
	S2650/mda	2p25; 3p26; 5p15.3-p13.2; **7p13-q11.2**; 8p23-p22; 13q21; 13q32-q34; 15q26; 18q22-q23	10p15-q26; 17q21; Xp22.3-q28
	GBM/C1760/mda	1p36.3; 2p25-p24; 3p26; 3q28-29; 4p16; 4q34; 5p15.3;	6q11.1-q22; 9p23-p12; 10p14-q25; 16p13.3
	GBM/C1760/mda	1p36.3; 2p25-p24; 3p26; 3q28-29; 4p16; 4q34; 5p15.3; 5q35; 7p22-q36; 11p15; 11q24; 12p13; 13q33; 15q26; 18p11.3; 18q22-q23; 1 9q13.3, 20p13-p12; 20p11.2-q11.2; 20q13.2; 21q22	6q11.1-q22; 9p23-p12; 10p14-q25; 16p13.3

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		Metaphase CGH data		Array CGH data
	Tumour ID	Gains	Losses	Tumour ID
PRIMARY CLIOBLASTOMAS	S2093*#	18q23	None	The gain at 18q23, reported by metaphase CGH, was unsupported in the array. Instead, the array revealed gain of 3 clones on 7p two of which contain EGFR sequences. There is slight underrepresentation of the entire CHR 10
	GBM/C160*#	None	2q37	Essentially normal; the apparent loss reported by metaphase CGH was not substantiated.
	GBM/C1510#	7p15; 7p13; 7q21; 7q22-q32	9q34; 16q12.2; 19p13.3-p12	CHR 7 is slightly overrepresented throughout, CHR 10 slightly underrepresented throughout, while losses reported by metaphase CGH on 9q, and 19p are not substantiated.
	GBM/C1706*#	13q21; Xp22.1-q28	None	Essentially normal
	GBM/C1752*#	9q12	None	Essentially normal
	GBM/C1612/mda#	3p26-p14; 3p12-q11.2; 3q28; 4p15.2-p13; 6p25-q26; 7p22-q35; 8p22-q24.3; 21q11.2-q21; Xp22.3-q12; Xq13-q28	2p25-q37; 4q21-q22; 4q28-q35, 10p15-q26, 13q11-q34, 14q11.1-q12; 16p13.3; 17p11.2; 19p13.3; 19p13.2	Several clones are prominently deleted between 85-90 MB, (corresponding to 10q23) and at 115-120 MB (corresponding to 10q25-q26). Details discussed in Chapter 4
RECURRENT GLIOBLASTOMAS	GBM/C1724#	1p22; 4p15.1-p11; 5p14-p12; 8p11.2-q24.3; 9p12-q33; 21q11.1-q22	2q35-q37; 11q22-q25; 13q32-q34; 14q24-q31; 16p13.3-q24	The set clones deleted in CHR10 of 1612 were also deleted in 1724. In CHR17, a few clones were underrepresented around 10MB, corresponding to the location of TP53.
	GBM/C1724/mda	1p31-p13; 1q22-q23, 4p15.3-p12; 8p11.2-q24.3; 9p13-q11; 9q13-q34; 21q11-q22	11p11.2-q11; 11q22-q25; 13q21-q22; 13q33-q34; 14q21-q24; 16p13.3-q23	
	GBM/S2687/mda	5p15.3; 7p22-q11.2; 7q21-q36	10p15-p11.1; 10q21-q24; 15q21-q23; 16p13.2-p12; Xp22.1-q28	

*, Tumours that had <3 CNAs (copy number alterations) in the autosomes (n=5); #, tumours that were further investigated by array CGH (n=7)

Appendix 1 cont..

With the exception of 7 tumours, all 33 gained and/or lost at three separate chromosome regions. Each of the 8 tumours showed either gains or losses only and generally had fewer CNAs. Three had no gains: AA24 showed three loss-CNAs, GBM/S11 lost in 10 separate regions, while GBM/C160 only lost at 2q37. Six remaining tumours had no losses overall; of these 4 gained in only one region (GBM/S2093; 18q23), GBM/S2848; Xp22.3-q28, GBM/C1752; 9q12, and GBM/C2394; Xp22.2-q27 while two lost in two regions, GBM/C1706 at 13q21 and Xp22.1-q28; and GBM/C2685 at 15q11.2-q12 and Xp22.2-q12 respectively. Bold font, regions that are markedly gained or lost; light green colour shading, tumours with 0-3 CNAs in autosomes; Gray-shaded areas, regions of (or bordering) heterochromatin. **a+** and **a-** (respectively representing gains and losses), show specific alterations revealed by array CGH which were unreported in the metaphase experiments.

Appendix 2: Recurrent regions of overlap of CNAs in a panel of 26 tumours investigated

Copy number losses: MCRO	Frequency %	Copy number gains: MCRO	Frequency %
16q11.1	50	7p13-14.1	50
16p13.3-p13.2	46	7p15.1-p15.3	50
19p13.2-p13.3	46	7q22	50
19p13.13	42	7q31	50
19p13.12-p13.1	42	7q21	46
22q13.2-q13.32	42	13q21.1-q21.33	39
22q12.2-q13.2	38.5	1p13.2-p31.2	31
10q25.1-q26.12	38.5	12q14.3-q21.2	31
10p11-21	35	1p22	27
10q11.21-q11.22	35	5q23.3	25
16p13.3-p12.2	35	2p24.3-p25.3	23
19q13.2-q13.42	35	3p25.3-p26.3	23
10p11.23-p12.2	31	4q26-q27	23
10q23.1-q23.33	31	5p15.2-p15.33	23
19q13.11-q12.13	31	5q14-q21.2	23
9q34.1-q34.2	27	7p22.1-p22.3	23
10q21.1-q21.3	27	13q21-q22	23
17p13.1-p13.3	27	13q33.1-q33.33	23
19q12	27	2q22-q23	19
22q11.21-q12.1	27	2q31.2-q31.3	19
1p34.1-p35.3	23	3p12	19
2q35-q37.3	23	4p14-p15.1	19
10p12.31-p15.1	23	5q21.2-q22.2	19
14q24.1-q32.32	23	6p25.1-p25.2	19
16q12.2-q13	23	6q12	19
17p11.2-p12	23	6q22	19
1p36.1-36.23	19	8q22-q23	19
1q12	19	9p24.2-q24.3	19
13q33.1-q34	19	11q23.2-q24.3	19
15q11.1-q12	19	15q25.1-q26.2	19
16q22.3-q24.2	19	18p11.32	19
11q12.2-q13.1	15	20p13	19
17q21.1	15	21q11.2-q21.2	19
20q12-q13.2	15	21q22.13-q22.2	19
9p12-p13.2	11.5	4q22-q24	15
13q13.3-q14.12	11.5	4q11-q13.3	15
17q11.2-q12	11.5	6q15-q16.2	15
17q22-q23	11.5	8p23.3	15
		12p13.32-p13.33	15
		20p12.32-p12.3	15
		3q13.11-q13.12	11.5
		9p21.1-p21.3	11.5
		9q13-q33.3	11.5
		11p15.5	11.5
		11q14.1-q14.3	11.5

Appendix 2: A list of 84 MCRO (minimum common regions of overlap) of CNAs observed in at least 3 out of 26 high-grade astrocytomas investigated in this study.

Appendix 2

Frequencies are expressed as a percentage. The chromosomal regions in which the most frequent losses are found implicate loci of probable tumour suppressor genes, DNA repair genes, and landscapers that may be involved in the pathogenesis of astrocytomas. In some cases massive deletions span the entire chromosome arm or the whole chromosome. Chromosome studies have revealed many deletions, which point to the presence of tumour suppressor genes or DNA repair genes at corresponding regions. For some examples, see Appendix 3.

Appendix 3: Comprehensive list of CNAs in the study with sample candidate genes mapped in respective loci

	Number of Informative tumours		Frequently altered chromosome region				Genes of Interest
	Total N=34	p/q	Gains	Cases	Losses	Loss	
Chr.1	15	15/8	1p13.2-p31.2 (1p22),1q31	8(7), 2	1p31.3-p36.3 (1p35.1-p35.3), 1q12,	8(6), 5	EPHB2, PAX7, BMP8B, MAP3K6, WNT2B
Chr.2	15	7/11	2q21.1-q33.3 (2q22-q23; 2q31.2-q31.1)	6(5;5)	2p24.3-p25.3(2p25), 2q35-q37.2(2q37)	6(6), 6(6)	DDX1, NMI, IGFBP5, PAX3, ERBB4, PMS1, NEUROD1
Chr.3	12	11/6	3p25.3-p26.3, 3p12, 3q13.11-q13.12, 3q25.31-q25.33, 3q29	6, 5, 5, 2, 1	3p14-p21, 3q26.3	1, 1	MSH1, RARB, ROBO1, EIF4, EPH3 TP73
Chr.4	12	8/10	4p14-p15.3, 4q22-q24, 4q26-q27	5, 5, 7	4p16.2-p16.3, 4q21.1-q21.3	1, 1	SLIT2, PROM1/CD133, FGFR3, MSX1, NKX3-2, BAPX, NKX6, PRGFC, VEGF/KDR, SMAD1, EGF, EMCN
Chr.5	14	8/10	5p15.2-p15.33, 5p11-p14.3, 5q14-q23, 5q21.2-q22.2, 5q23.3	6, 2, 6, 5, 3	5q15.33, 5q35.1	1, 1	PC4, SEMA5A, GTF2H2, FST, ISL1, CCNH, CDC25C, EFNA5, NEUROG1, FGF, PDGFR
Chr.6	10	5/7	6p25.1-p25.3, 6q12, 6q15-q16.2, 6q22	6, 5, 4, 5	6q11.1-q22	1	VEGF, EPH7, FIG, CCNC, PEX7, CDK11, HEY2, ATXN, TNFAIP3, MAP3K5, OLIG3, NMBR, BLBP, TCF21
Chr.7	17	11/15	7p13-p14, 7p15.1-p15.3, 7q21, 7q22, 7q31		None	N/A	HOXA1, HOXA13, PDGF, EGFR, RAC1, GBAS, PHKG1, HGF/SF, HGFR/MET, PI3K, FRA7G, N-WASP/WASL, SMO
Chr.8	10	7/7	8p23.3, 8q22-q23 (8q22)	4, 7(3)	8p12-p23	1	SCA/BHLH.2, NRG1, NKX3-1, CCNE2, E2F5
Chr.9	22	13/14	9p21.1-p21.3, 9p24.2-p24.3	3, 5	9p21.1-p21.3, 9q34.11-q34.2	3, 7	CDKN2AandCDKN2B, IFNB1, MTAP, DMRTA1, SCA1A1, PTCH, ASTN2, FIBCD1, TSC1, ABL1, DAPK1
Chr.10	14	12/12	10p15.1-p15.3	2	10p11.21, 10p11.22-p11.23, 10q21.2-q21.3, 10q23.1-q23.3, 10q25.1-q26.1	9, 8, 6, 8, 10	PTEN/MMAC1/SLIT1, PAX2, MGMT, NKX6-2, VAX1, SUFU
Chr.11	12	7/12	11p15.5, 11q14-q14.3, 11q23.2-q24.3	3, 3, 5	11p15.2-p15.5, 11p12-q11, 11q12.2-q13.1	2,2, 4	TFIIH1, ASCL3, MYOD1, ESP10, HRAS, FLJ23311, MAP4K2, VEGFB, PAX6, CD44, CCND, CTNND1, PRG2andSLC43A

Appendix 3 cont...

	Number of Informative tumours		Frequently altered chromosome region				Genes of Interest
	Total N=34	Chr, arm p/q	Gains	Cases	Losses	Loss	
Chr.12	12	6/8	12p13.32-p13.33, 12p11-p12, 12q14.3-q21.2	4,2, 8	12q24.1-q24.3	2	MGST1, NTF3, GAPD, CDKN1B*, ENO2, CDK2, CDK4, MDM2, SAS, BRAP, MAPKAPK9, GRF2H3, BCL2L14*
Chr.13	18	18	13q13.3-q14.12, 13q21.1-q22.3(13q21.1-q21.3), 13q33.2-q33.3	3, 9(8), 4	13q13-q14, 13q33.1-q33.3	3, 5	LATS1, IBPF1, CDK6, RB1, DDX26
Chr.14	7	7	None	N/A	14q24.2-q32.33(14q31-q32.32)	7(7)	TIF1, NKX2-9, MAP4K5, BMP5, V-AKT/AKT1, MLH3*, CDC42BPB, PTPN1, SEL1L*, SERPINA2*
Chr.15	9	9	15q26	5	15q11.1-q11.12, 15q21.2-q22.32	5, 1	CRABP1, NEO1, FES, MESP1
Chr.16	19	14/13	None	N/A	16p13.2-p13.3, 16p12.3-p13.12, 16q11.2, 16q11.2-q12, 16q23.3-q24.1	12, 9, 13, 6, 5	MRPL28, AXIN1, TSC2, MLCK, LOC440374, RABL2, MAP3K7, ARID3A, FRA16D, CDK10, MMP2*, FANCA*, STYWOBU*
Chr.17	17	10/9	17p13.3	1	17p12-p13, 17q21.1, 17q24.1-q25.1	7, 4, 6	TP53, EIF4A1, HES7, NEUROD2, ERBB2, GFAP, BRCA1, IGI35, HOXB, CDK3, MAP2K6, SYM1, SYNS1, MRC2*
Chr.18	9	5/6	18p11,3, 18q22.2	5, 5	None	N/A	RAB31, SMAD7, DCC.1
Chr.19	15	13/11	19q13.31-q13.33	1	19p13.2-p13.3, 19p13.2-p13.3	13/10	NHLH2, CDKN2D, ELAV2, CCNE, SERTAD1, TGFB1, NTF5, APC2*, BAX*, ELSPBP1*
Chr.20	10	6/6	20p12.1-p12.3, 20p13, 20q11.3-q13.2	6, 5, 2	20q13.2, 20q12-q13.11	4, 2	NXT1, SOX12/SOX20, NKX2-2, CDC25andC20orf25, SMOX, E2F, CHD5/CHD6, BCAS4, MYBL2, MMP9*
Chr.21	7	7	21q11.2-q21.2, 21q22.13-q22.2	3, 6	21q22.12-q22.13	1	S100B, OLIG2, GRIK1, H2B, WDRandSH3BGR, RUNX1
Chr.22	12	12	None	N/A	22q11.21-q12.1, 22q12.3-q13.32	7, 12	NF2, HGFL (MGC17330), KREMEN1, LIMK2, CHEK2, BCR, BCL2L13*

Legend for appendix 3:

Genes that are listed in table 6.4 participate in various cellular functions, for example proliferation, control of the cell cycle and apoptosis, while others have been included because of known crucial roles in neurogenesis. * Genes with MMR (or caretaker) functions (genes belonging to one of two categories known to protect cells from chromosomal instability). Aberrations in these genes commonly predispose cells to malignancy. Table 6.5: summarizes the frequencies of occurrence of DNA copy number alterations in regions that harbour established mismatch repair (MMR) (mutators) in this study.

Appendix 4 - Clones altered in GBM/C160

CHR position (Mb)	Clone	Fluorescence ratio (Mean)		Matching genes (locus)
		Test/Ref	Control	
CHR1_25	CTB-14E10	2.09,2.25		No record
CHR2_89.77	RP11-316G9	0.70,0.75	1.07	None
CHR5_131	CTB-104P14	2.04,2.00	0.99	SLC22A4 (5q23.3)
CHR5_175.8	CTB-87L24	1.30,1.32	0.97	RNF44 (5q35.2); ETEA (5q35.2)
CHR6_32.5	RP1-93N13	1.27,1.24	0.69	None
CHR6_169	RP1-137D17	1.78,1.99	N/A	None; clone mapped to 6q27
CHR7_98.5	RP4-550A13	1.27,1.35	0.96	ARPC1A (7q22.1)
*CHR8_16.29	RP11-19N21	1.25,1.22	0.97	6 genes, 5 of them clustered at 11.55 (CDKN1C, KCNQ1, SLC22A18, SLC22A1LS, PHLDA2), and the other at 11.54 (KCNQ1DN)
CHR11_118.8	CTD-3245B9	1.90,1.93	0.86	N/a; some STS mapped to CHR-X
CHR12_133.3	CTC-221K18	1.68,1.66	0.61	N/a; clone not in NCBI registry
CHR14_19.74	RP11-2F9	0.54,0.56	1.80	N/a
CHR14_105.12	CTC-820M16	1.22,1.23	1.21	No record
CHR15_99.4	RAP11-299G20	1.31,1.24	0.87	No record
CHR16_69.95	RP11-296I10	0.65,0.67	1.06	4 genes, 3 clustered at 16q22.1 (EXOSC6, MGC34761, PDPR), and one at 16q22 (AARS)
CHR19_9.10	CTC-2547N9	1.44,1.46	N/A	No record
CHR19_9.10	CTC-444D3	1.62,1.63	1.04	6 genes, 6 clustered at 19p13.2 (OR7D4, OR7G1, OR1M1, OR7D2, OR7G2, OR7G3), and one at 19p13 (ZNF317)
CHR19_13.14	CTC-250I14	1.65,1.52	0.87	4 genes, 3 of them clustered at 19p13.13 (BTBD14B, STX10, IER2), and one: CACNA1A and 19p13.2-p13.1.
CHR19_48.48	CTC-490G23	1.88,1.97	1.11	5 genes, 4 all mapped to 19q13.2 (PSG4, PSG5, PSG9, PRV1), and one: MGC4766 at 19q13.31.
CHR19_52.4	CTC-483I11	2.07,2.17	0.88	3 genes, 2 are mapped to 19q13.32 (SAE1, CCDC9), and one (BBC3) is at 19q13.3-q13.4

Appendix 4 – Clones altered in GBM/C1510

CHR position (Mb)	Clone	Fluorescence ratio (Mean)		Matching genes (locus)
		Test/Ref	Control	
CHR5_14.54	RP1-29-29O12	0.59,0.59	1.36	None
CHR6_32.58	RP1-93N13	0.71,0.68	0.70	None
CHR7_38.01	RP11-121A8	1.30	1.01	STARD3NL (7p14-p13)
CHR7_86.75	CTB-1137N13	1.28	1.01	Clone not found in NCBI Clone Registry
CHR7_123.39	RP5-902E20	1.30	1.04	None
CHR7_136.51	RP11-8P6	1.30	n/a	Mapped to CHR 18, & X; No genes
CHR9_65.21	RP11-265B8 (1/2)	*0.48 (0.96)	0.97	3 genes found: FXN at 9q13-q21.1, others (PRKACG & PIP5K1B) are both at 9q13
CHR11_118.80	CTD-3245B9	1.33,1.35	0.86	N/a; some STS mapped to CHR-X
CHR12_133.30	CTC-221K18	1.25,1.25	0.61	N/a; clone not in NCBI registry
CHR15_19.74	RP11-2F9	0.52,0.52	1.80	N/a
CHR16_69.95	RP11-296I10	0.71,0.70	1.01	4 genes, 3 clustered at 16q22.1 (EXOSC6, MGC34761, PDPR), and one at 16q22 (AARS)
CHR17_43.73	RP5-1169K15	0.65,0.66	1.06	MAP3K14 (17q21)
CHR19_8.58	CTD-2547N9	1.27,1.34	0.84	N/a

Appendix 4 - Clones altered in GBM/C1706

CHR position (Mb)	Clone	Fluorescence ratio (Mean)		Matching genes (locus)
		Test/Ref	Control	
CHR1_0.25	CTB-14E10	1.65	0.48	None
CHR1_172.12	RP5-1045J21	1.48	0.92	N/A; mapped to 1q23.3-1q24.3
CHR2_89.77	RP11-316G9	0.71	1.19	N/a; mapped to multiple CHRs: 2, 4, 12 and X
CHR2_190.07	CTC-444N24	1.30	0.66	5 genes, 3 mapped to 19q13.4 (ZNF272, ZNF304, ZNF264) and 2 to 19q13.43 (ZNF543, AURKC).
CHR5_69.35	RP11-497H16	0.72	1.20	N/a; multiple mappings: 6p25, 5q12, 5p
CHR5_110.12	CTC-534K6	1.43	0.91	N/a; mapped to 5q13-5q14
CHR5_127.68	CTC-352M6	1.37	1.05	FBN2 (5q23-q31); at least one STS of the primary CHR 5 Contig. was mapped to CHR-X
CHR5_131.68	CTB-104P14	1.51	0.89	SLC22A4 (5q23.3)
CHR6_32.58	RP1-93N13	0.79	1.20	N/a
CHR11_118.80	CTD-3245B9	1.51	0.80	N/a; some STS mapped to CHR-X
CHR12_37.92	RP11-462G12	1.22	1.01	ADCY9 (16p13.3); an STS mapped to CHR 3
CHR12_133.30	CTC-221K18	1.45	0.66	N/a; clone not in NCBI registry
CHR15_19.74	RP11-2F9	0.72	1.03	N/a
CHR16_69.95	RP11-296I10	0.71	1.12	4 genes, 3 clustered at 16q22.1 (EXOSC6, MGC34761, PDPR), and one at 16q22 (AARS)
CHR17_43.73	RP11-374N3	0.71	0.82	N/a; multiple mappings, also on CHR 11
CHR19_5.06	CTC-482H14	1.49	0.83	JMJD2B (19p13.3)
CHR19_8.58	CTD-2547N9	1.52	0.54	N/a
CHR19_9.10	CTC-444D3	1.52	1.05	6 genes, 6 clustered at 19p13.2 (OR7D4, OR7G1, OR1M1, OR7D2, OR7G2, OR7G3), and one at 19p13 (ZNF317)
CHR19_13.14	CTC-250I14	1.44	0.76	4 genes, 3 of them clustered at 19p13.13 (BTBD14B, STX10, IER2), and one: CACNA1A and 19p13.2-p13.1.
CHR19_33.30	CTD-2043I16	1.24	0.82	N/a; multiple ePCR mapping, also on CHR 14
CHR19_44.45	CTC-246B18	1.35	0.95	6 genes, 3 mapped to 19q13.13 (IL28A, IL28B, IL29) and 3 at 19q13.2 (FLJ10211, LRFN1, GMFG)
CHR19_48.48	CTC-490G23	1.38	0.96	5 genes, 4 all mapped to 19q13.2 (PSG4, PSG5, PSG9, PRV1), and one: MGC4766 at 19q13.31.
CHR19_52.40	CTC-483I11	1.48	0.84	3 genes, 2 are mapped to 19q13.32 (SAE1, CCDC9), and one (BBC3) is at 19q13.3-q13.4

Appendix 4 - Clones altered in GBM/C1752

CHR position (Mb)	Clone	Fluorescence ratio (Mean)		Matching genes (locus)
		Test/Ref	Control	
CHR2_89.71	RP11-316G9	0.71	1.19	N/a; multiple mapping, also on CHRs 4, 12, and X
CHR2_180.28	RP11-391P1	1.33	1.00	N/a
CHR2_190.07	CTC-444N24	1.26	0.66	5 genes, 3 mapped to 19q13.4 (ZNF272, ZNF304, ZNF264) and 2 to 19q13.43 (ZNF543, AURKC).
CHR2_205.07	RP11-507C18	1.25	0.83	N/a
CHR4_133.92	RP11-149A7	1.39	0.65	N/a; mapped by FISH to "4q28.3b" and 4q27
CHR5_10.40	CTD-2274H20	1.30	0.60	N/a; mapped to 5p15-5p14
CHR5_55.38	RP11-412L4	0.78	0.93	N/a; mapped to CHR 5 & 12
CHR5_69.35	RP11-497H16	0.78	1.20	N/A; multiple mapping, to 6p25, 5q12, 5p
CHR5_127.68	CTC-352M6	1.29	1.05	FBN2 (5q23-q31); at least one STS of the primary CHR 5 Contig. was mapped to CHR-X
CHR5_131.68	CTB-104P14	1.34	0.89	SLC22A4 (5q23.3)
CHR6_20.14	RP11-86O17	1.33	0.83	N/a
CHR7_103.76	RP11-148A10	1.25	0.87	N/a
CHR10_45.52	RP11-432I13	1.25	0.99	OR13A1 (10q11.21); also mapped on CHR 12
CHR10_64.09	RP11-13A2	1.39	0.86	ZNF365 (10q21.2)
CHR10_126.73	RP13-238F13	1.28	0.91	3 genes: 2 (C10orf121 & LHPP) mapped to 10q26.13, and OAT is at 10q26
CHR11_118.80	CTD-3245B9	1.49	0.80	N/a; some STS mapped to CHR-X
CHR11_123.88	RP11-485A5	1.48	1.01	N/a
CHR12_52.53	RP11-96P3	1.78	0.78	N/a
CHR12_95.62	RP11-74K11	0.80	1.12	PLXNC1 (12q23.3)
CHR12_133.30	CTC-221K18	1.53	0.66	N/a; clone not in NCBI registry
CHR15_46.71	RP11-485O10	1.45	0.76	3 genes: Cep152 (15q21.1) while CRI1 & RaLP are mapped to 15q21.1-q21.2
CHR16_49.48	RP11-305A7	0.77	0.99	N/a; mapped to 16q12.1
CHR16_69.95	RP11-296I10	0.72	1.09	4 genes, 3 clustered at 16q22.1 (EXOSC6, MGC34761, PDPR), and one at 16q22 (AARS)
CHR17_69.53	RP11-144K9	1.25	0.74	N/a
CHR18_41.77	RP11-486C18	1.32	0.81	N/a
CHR19_8.58	CTD-2547N9	1.50	0.54	N/a
CHR19_9.10	CTC-444D3	1.33	1.05	6 genes, 6 clustered at 19p13.2 (OR7D4, OR7G1, OR1M1, OR7D2, OR7G2, OR7G3), and one at 19p13 (ZNF317)
CHR19_13.14	CTC-250I14	1.29	0.76	4 genes, 3 of them clustered at 19p13.13 (BTBD14B, STX10, IER2), and one: CACNA1A and 19p13.2-p13.1.
CHR19_40.95	RP11-38C1	1.24	0.60	N/a
CHR19_54.60	RP11-521I20	1.36	0.85	N/a; also mapped to CHR 6
CHR22_45.36	CTA-29F11	1.25	0.91	2 genes: C22orf4 (22q13.3) and CERK (22q13.31).
CHR-X_0.54	CTB-98C4	1.23	0.63	No record

CHR-X_2.22	RP11-457M7	0.80	0.94	N/a; multiple mapping to X, Y and CHR 5
CHR-X_10.66	RP1-27C22	1.89	0.74	2 genes: AMELX (Xp22.31-p22.1) and ARHGAP6 (Xp22.3)
CHR-X_149.19	GS1-225F9	0.80	N/a	No record

Appendix 4 - Clones altered in GBM/S2093

CHR position (Mb)	Clone	Fluorescence ratio (Mean)		Matching genes (locus)
		Test/Ref	Control	
CHR1_0.25	CTB-14E10	1.51	0.48	No record
CHR1_9.80	RP4-575L21	0.73	1.07	UBE4B (1p36.3); also maps to CHR 12
CHR1_28.40	RP4-669K10	0.74	0.97	5 genes, 4 of which map to 1p35.3 (MGC45806, SECP43, TAF12, LOC85028) and one (CHC1) maps to 1p36.1
CHR1_69.54	RP5-944F13	1.29	0.78	N/a; clone maps to 1p31.2-1p32.1
CHR1_112.68	RP11-31F15	0.72	0.99	N/a; clone maps to 1p13.1-1p13.3, and also to CHR 12
CHR1_221.18	RP11-100E13	0.76	1.04	FLJ38993 (1q42.12)
CHR4_162.05	RP11-502F14	1.38	0.68	N/a
CHR5_14.54	RP1-29O12	0.64	0.99	N/a
CHR5_69.35	RP11-497H16	0.65	1.20	N/a; multiple mappings: 6p25, 5q12, 5p
CHR5_110.12	CTC-534K6	1.42	0.91	N/a; mapped to 5q13-5q14
CHR5_131.68	CTB-104P14	1.44	0.89	SLC22A4 (5q23.3)
CHR6_32.58	RP1-93N13	1.28	1.20	None
CHR6_155.24	RP1-66H9	0.75	1.20	N/a; also maps to CHR 2
CHR6_169.36	RP1-137D17	1.55	0.75	N/a; clone mapped to 6q27
CHR7_54.43	RP11-449G3	3.28	0.81	N/a
CHR7_54.85	RP5-1091E12	5.02	1.00	EGFR (7p12)
CHR7_55.01	RP11-339F13	3.99	1.05	EGFR (7p12); FISH maps clone to 7p11.2-p12
CHR8_132.15	RP11-4C17	0.77	0.82	N/a
CHR9_6.65	RP11-106A1	0.68	1.21	GLDC (9p22); also maps to CHR 12
CHR9_23.46	RP11-495L19	0.68	0.92	*ePCR maps this clone to CHR-X
CHR9_27.50	RP11-27J8	0.64	1.17	3 genes, all mapped to 9p21.2 (C9orf72, IFNK, MOBKL2B)
CHR10_12.01	RP11-401F24	0.64	1.22	3 genes 2 (C10orf47 & ECHDC3) mapped to 10p14, and UPF2 mapped to 10p14-p13
CHR10_29.17	RP11-478H13	0.67	0.95	N/a
CHR10_45.98	RP11-534N5	0.67	1.12	KIAA0592 (10q11.1); sequence also maps to CHR 12
CHR10_74.10	RP11-326F3	0.67	1.05	N/a
CHR10_101.92	RP11-724N1	0.68	1.01	CNNM2 (10q24.33); sequence also mapped to CHR 19
CHR10_133.73	RP11-45A17	0.71	1.08	BNIP3 (10q26.3); clone also maps to CHR-X
CHR11_14.09	RP11-502G22	0.76	0.87	SPON1 (11p15.2)
CHR11_118.80	CTD-3245B9	1.44	0.84	N/a; some STS mapped to CHR-X
CHR11_133.06	RP11-545G18	0.77	N/a	N/a
CHR12_113.06	RP11-495K9	0.76	0.87	SFRS8 (12q24.33); also maps to CHRs 13, 5, and 12
CHR12_113.30	CTC-221K18	1.27	0.66	N/a; clone not in NCBI registry
CHR13_103.35	RP11-502J9	0.79	0.78	N/a
CHR14_21.45	RP11-98N22	0.73	0.75	2 genes both mapped to 14q11.2 (OR4K17, OR4N5)
CHR14_22.55	RP11-298I3	0.71	1.18	8 genes: one (SKB1) mapped to 14q11.2-q21, and 7 all clustered at14q11.2 (ACIN1, C14orf94, RBM23, PSMB5, C14orf93,

				CDH24, JUB)
CHR14_32.89	RP11-500A9	0.76	0.97	* This clone is mapped to CHR 12 in the NCBI clone registry
CHR15_19.74	RP11-2F9	0.55	1.03	N/a
CHR15_20.57	RP11-13O24	0.76	1.45	N/a
CHR15_38.64	RP11-532F12	0.74	0.81	7 genes: GCHFR mapped to 15q15, while all six others are at 15q15.1 (PPP1R14D, FLJ10579, FLJ10634, RAD51, SPINT1, ZFYVE19)
CHR15_82.77	RP11-565O12	0.77	1.03	SPC18 (15q25.3)
CHR16_0.21	CTB-121I4	0.75	0.84	No record found in the NCBI cone registry
CHR16_11.99	RP11-31O11	0.68	0.93	N/a
CHR16_16.10	CTD-2504F3	0.73	1.15	ABCC1 (16p13.1)
CHR16_22.61	RP11-105B19	0.73	1.03	N/a; also mapped to CHR 5
CHR16_29.73	RP11-74E23	0.76	1.04	N/a; mapped to 16p11.2
CHR16_69.95	RP11-296I10	0.57	1.39	4 genes, 3 clustered at 16q22.1 (EXOSC6, MGC34761, PDPR), and one at 16q22 (AARS)
CHR17_7.52	RP11-199F11	0.75	1.12	4 genes including TP53, FLJ10385, FLJ46675, which are all mapped to 17p13.1, and one - EFNB3 – which maps to 17p13.1-p11.2
CHR17_29.20	RP11-229K15	0.78	1.12	N/a
CHR17_39.93	RP11-156E6	0.78	1.15	6 genes of which only ACLY maps to 17q12-q21, while 5 others are all clustered at 17q21.2 (SC65, MGC20781, KLHL10, KLHL11, FKBP10, DKFZP434H0115)
CHR17_43.73	RP11-334N3	0.69	N/a	N/a
CHR17_80.04	RP11-525L23	0.75	0.93	N/a
CHR17_81.23	RP11-567O16	0.72	0.77	TBCD (17q25.3)
CHR19_5.06	CTC-482H14	1.47	0.83	JMJD2B (19p13.3)
CHR19_13.72	RP11-285H8	0.73	0.91	N/a; clone also maps to CHR 5
CHR19_38.17	RP11-298M15	0.69	1.09	N/a
CHR19_52.40	CTC-483I11	1.41	0.84	3 genes, 2 are mapped to 19q13.32 (SAE1, CCDC9), and one (BBC3) is at 19q13.3-q13.4
CHR19_52.98	CTD-2571L23	0.71	0.72	5 genes, FLJ40321 mapped at 19q13.32, the others at 19q13.3 (CRX, GLTSCR2, EHD2, SEPW1)
CHR19_54.05	CTD-2639E6	0.72	0.91	10 genes of which 2 are mapped to 19q13.3-q13.4 (BAX, 19q13.3-q13.4), 2 others are mapped to 19q13.3 (DHDH, GYS1), another 2 mapped to 19q13.33 (DHRS10, PLEKHA4). 4 remaining genes are mapped variously, PPP1R15A at 19q13.2, NUCB1 at 19q13.2-q13.4, BCAT2 at 19q13, and TULP2 at19q13.1
CHR20_30.95	RP5-857M17	0.78	1.13	3 genes, ID1 mapped to 20q11, while BCL2L1 & COX4I2 are both at 20q11.21
CHR20_45.13	RP3-337O18	0.75	1.22	8 genes, one (PPGB) mapped to 20q13.1; 2 mapped to 20q12-q13.1 (PTE1, PLTP) and all remaining five mapped to 20q13.12 (C20orf165, NEURL2, ZSWIM3,

				C20orf161, ZSWIM1).
CHR22_38.36	CTA-229A8	0.78	0.95	2 genes: GPR24 (22q13.2) and MKL1
				22q13
CHR22_39.70	RP11-422A16	0.78	1.08	EP300 (22q13.2)
CHR22_40.60	CTA-250D10	0.77	0.99	7 genes: NAGA at 22q11, SREBF2 at 22q13, TNFRSF13C at 22q13.1-q13.31, and 4 at 22q13.2 (LOC150368, MGC26816, SEPT3, C22orf18)
CHR-X_43.55	RP11-386N14	0.79	1.13	2 genes: DUSP21 at Xp11.4-p11.23 and UTX at Xp11.2

Appendix 4 – Sample clones altered in CHR1 of GBMs 1612 and 1724

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref)			Matching genes (locus)
		1612	1724	CTRL	
CHR1_66.89	RP11-412F21	1.07	1.22	1.09	N/a: work abandoned
CHR1_142.71	RP11-326G21	1.24	1.40	1.06	PDE4DIP (1q12)
CHR1_225.32	RP4-799G3	1.28	1.03	0.92	N/a; mapped to 1q42.11-1q42.3
CHR1_232.19	RP11-509I1	1.24	1.19	0.96	2 genes: CHS1 (1q42.1-q42.2) & GNG4 (1q42.3)
CHR1_44.17	RP11-184I16	1.04	1.25	0.98	2 genes: SIAT6 () & JMJD2A (1p34.2-p34.1). STS(s) from this clone also map to CHR 7
CHR1_217.78	RP11-528D17	0.72	1.00	0.92	N/a
CHR1_221.18	RP11-1000E13	0.65	0.93	0.94	No record of this clone in NCBI clone registry

Appendix 4 – CHR2

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref)			Matching genes (locus)
		1612	1724	CTRL	
CHR2_89.12	RP11-294I20	0.61	0.77	1.15	N/a: clone is mapped to CHR 19 on the NCBI clone registry
CHR2_89.77	RP11-316G9	0.50	0.73	1.12	N/a; STSs from this clone also map to CHRs 4, 12 & X
CHR2_131.89	RP11-209H16	0.63	0.76	0.99	2 genes: ARHGEF4 (2q22) & PLEKHB2 (2q21.1)
CHR2_206.40	RP11-325M10	0.63	0.79	0.99	ALS2CR19 (2q33.3)
CHR2_241.01	RP11-299H21	1.82	0.84	1.05	2 genes, both mapped to 2q11.2 (RNF149, C2orf29)
CHR2_22.62	RP11-368O18	0.68	0.74	1.01	N/a
CHR2_130.68	RP11-32C20	0.66	0.77	1.11	N/a; The clone is mapped to q214.3. a STS from this clone also maps to CHR 7

Appendix 4 – CHR3

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR3_52.52	RP11-447A21	0.74	0.94	1.07	5 genes of which PB1 is mapped to 3p21 and the other four (NS, SPC12, AD-017, NEK4) are all clustered at 3p21.1
CHR3_65.15	RP11-88H12	1.34	1.00	0.97	BAIAP1 (3p14.1)
CHR3_86.93	RP11-81P15	1.34	1.03	0.96	FLJ38507 (3p12.1)
CHR3_144.73	RP11-165M11	1.35	1.02	0,95	SLC9A9 (3q24)
CHR3_153.04	RP11-64O13	0.78	1.08	0.94	N/a
CHR3_170.37	RP11-141C22	1.46	1.04	1.00	N/a; clone mapped to 3q26.1
CHR3_194.55	RP11-552A14	0.73	0.90	1.04	N/a
CHR3_195.53	RP11-279P10	1.35	1.04	1.04	3 genes all mapped to 3q29 (LRRC15, GP5, AFURS1); some STSs map to multiple chromosomes, i.e. CHR12 and 14.

Appendix 4 – CHR4

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR4_1.16	RP11-296G16	0.66	0.72	0.98	N/a
CHR4_8.11	RP11-338K13	0.67	0.81	0.96	3 genes: LOC389199 (4p16.1), AFAP (4p16), ABLIM2 (4p16-p15).
CHR4_13.65	RP11-341G5	1.34	1.68	0.98	N/a
CHR4_20.51	RP11-362J17	1.35	1.61	0.95	KCNIP4 (4p15.31)
CHR4_57.71	RP11-355L4	0.69	0.78	0.95	N/a; mapped to 4q12; one STS additionally maps to CHR 12
CHR4_62.67	RP11-24I7	1.26	0.89	0.97	LPHN3 (4q13.1)
CHR4_110.13	RP11-75N20	0.64	0.78	1.04	2 genes both of which map to 4q25 (AGXT2L1, COL25A1)
CHR4_117.87	RP11-55L3	0.65	0.75	0.94	N/a
CHR4_190.10	RP11-347K3	0.63	0.69	0.96	N/a

Appendix 4

Appendix 4 – CHR5

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR5_14.54	RP1-29O12	0.58	0.63	1.36	N/a
CHR5_17.69	RP11-556F3	0.69	0.83	1.03	N/a
CHR5_73.82	RP11-97L2	1.00	1.31	1.01	N/a; mapped to 5q12-5q13 (FISH)
CHR5_79.26	RP11-30D15	1.35	1.04	0.95	N/a
CHR5_84.95	CTC-480C2	1.22	0.97	0.95	N/a; mapped to 5q12-5q13 (FLpter)
CHR5_104.21	RP11-275K4	0.78	0.82	0.93	N/a
CHR5_110.16	RP3-502L6	*1.54	1.30	0.95	CTNND2 (5p15.2)
CHR5_145.25	CTC-370H24	0.76	1.09	0.98	2 genes both mapped at 5q32 (MGC21644, SH3RF2)
CHR5_178.48	RP11-281O15	1.44	1.03	1.05	4 genes to of which (GRM6, ZNF354C) are mapped at 5q32, while two others (ZNF454, FLJ21628) are mapped at 5q32.3

Appendix 4 – CHR6

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR6_32.58	RP1-93N13	1.70	1.35	0.70	N/a
CHR6_79.51	RP11-173D14	0.66	1.18	1.10	2 genes: IRAK1BP1 (6q14-q15), PHIP (6q14); some STSs also map to X-CHR.
CHR6_96.94	**RP11-4D24	0.69	1.07	0.97	All STSs map to CHR 4 NOT 6!
CHR6_155.95	RP11-100E6	0.90	0.75	1.11	STEF/TIAM2 (T cell invasion & metastasis 2); Clone could affect RAC pathway

Appendix 4 – CHR7

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR7_38.01	RP11-121A8	1.41	1.11	1.01	STARD3NL (7p14-p1)
CHR7_66.89	RP11-358M3	1.25	0.87	1.09	N/a
CHR7_80.75	RP11-28I21	1.37	1.04	1.05	N/a; some of the STSs map to CHR 1
CHR7_83.31	RP11-313H6	1.33	0.78	1.07	N/a
CHR7_90.22	RP5-1084H12	1.29	0.93	0.98	N/a
CHR7_113.81	RP11-126C19	1.39	1.02	1.15	N/a
CHR7_118.47	RP11-105B19	1.31	1.31	1.10	N/a
CHR7_125.91	**RP11-21K15	1.41	1.10	1.01	N/a; Problem clone – all STSs map to CHR5 & one additionally maps to X-CHR
CHR7_140.71	RP11-5894A10	0.97	0.86	0.96	3 genes: FLJ10842, FLJ40852 & SSBP1 (single-stranded DNA-binding protein 1), all mapped to (7q34)

Appendix 4 – CHR10

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR10_49.26	RP11-13E1	0.58	0.84	1.03	FRMPD2 (10q11.22)
CHR10_74.10	RP11-326F3	0.67	1.12	0.99	N/a
CHR10_85.79	RP11-219F10	0.54	0.78	1.04	N/a
CHR10_89.83	RP11-380G5	0.02	0.32	1.01	PTEN (10q23.3)
CHR10_89.75	RP11-165M8	0.11	0.32	0.93	2 clones both mapped to 10q23.31 (CFLP1, ATAD1)
CHR10_90.66	RP11-304I5	0.33	0.54	1.06	2 genes both mapped to 10q23.31 (ANKRD22, LIPL3)
CHR10_117.10	RP11-338L11	0.09	0.33	1.00	ATRNL1 (10q26)

Appendix 4

Appendix 4 – CHR11

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR11_22.56	RP11-34N19	1.39	1.01	0.95	N/a
CHR11_30.73	RP11-302O8	0.92	0.74	1.04	N/a
CHR11_46.37	RP11-164L18	0.69	0.90	0.84	N/a; one STS additionally maps to CHR 12
CHR11_61.45	RP11-286N22	0.87	0.78	0.91	9 genes: DDB1 (11q12-q13), SYT7 (11q12-q13.1), MGC:13379 (11q13.1), and all remaining six are mapped to 11q12 (MGC20446, FLJ32771, DKFZP586B1621, HSPC196, FLJ20487, FLJ12529)
CHR11_83.65	RP11-118L6	1.05	0.79	1.01	N/a
CHR11_126.44	RP11-432I22	0.98	0.72	0.97	N/a
CHR11_128.53	RP11-264E20	0.94	0.59	1.04	N/a
CHR11_131.54	PAC1064E20	0.94	0.69	1.01	No record at the NCBI clone registry

Appendix 4 – CHR12

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR12_72.12	RP11-181D11	1.30	0.87	0.92	N/a
CHR12_78.00	RP11-202G24	1.05	0.72	1.02	N/a
CHR12_111.95	RP11-162P23	0.75	1.03	1.10	3 genes: ALDH2 (12q24.2), ACAD10 (12q24.12), BRAP (12q24)

Appendix 4 – CHR13

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR13_26.01	RP11-570F6	0.66	0.77	0.92	WASF3 (13q12)
CHR13_56.65	RP11-279F15	0.91	1.22	0.78	FLJ40296 (13q21.1)
CHR13_69.26	RP11-436I5	0.70	0.76	1.11	KLHL1 (13q21)
CHR13_113.86	RP11-245B11	0.64	0.87	0.85	RASA3 (13q34)
CHR13_106.65	RP11-232K11	1.3	0.87		N/a

Appendix 4 – CHR14

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR14_22.55	RP11-468E2	0.65	0.86	0.96	16 genes: NRL (14q11.1-q11.2), REC8L1 (14q11.2-q12), and all 14 other are clustered at 14q11.2 (ISGF3G, TM9SF1, LOC161247, C14orf20, C14orf123, C14orf122, PCK2, RNF31, PSME1, PSME2, IPO4, WDR23, LOC90668, CPNE6)
CHR14_36.33	RP11-138H18	0.70	0.81	1.10	TTC6 (14q21.1)
CHR14_50.38	RP11-463J10	0.62	0.80	1.04	NID2 (14q21-q22), C14orf166 (14q22.1), GNG2 (14q21)
CHR14_67.41	RP11-204K16	0.71	0.91	0.99	RAD51L1 (14q23-q24.2)
CHR14_87.82	RP11-79J20	0.67	0.79	0.96	N/a; mapped to 14q32.1
CHR14_96.07	RP11-76E12	0.65	0.77	0.98	N/a
CHR14_99.29	**RP11-94D19	1.80	1.29	1.02	N/a; Problem clone – mapped to 3p14, and several clones additionally map to CHR-X

Appendix 4 – CHR16

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR16_11.99	RP11-31O11	0.89	0.75	0.97	N/a
CHR16_22.61	RP11-105B19	0.89	0.70		N/a; one STS additionally maps at CHR5
CHR16_47.71	RP11-523L20	0.71	0.81	0.95	N/a
CHR16_49.48	RP11-452G23	0.99	0.82	1.01	N/a; mapped to 16q12.1. Some STSs additionally map to CHR-X
CHR16_53.70	RP11-357N13	0.90	0.70	1.03	FTO (16q12.2)
CHR16_68.22	RP11-76H6	0.92	0.69	0.93	N/a
CHR16_69.95	RP11-296I10	0.68	0.65	1.01	4 clones: AARS (16q22) and three clustered at 16q22.1 (EXOSC6, MGC34761, PDPR)

Appendix 4 - CHR17

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
***CHR17_7.518**					**Location of TP53 in relation to clones found in this array slide (plate)**
CHR17_7.52	RP11-31O11	0.42	0.58	0.85	N/a; Problem clone – all STSs mapped to CHR 5 and none to this chromosome
CHR17_7.65	RP11-404G1	0.39	0.49	0.96	FLJ46675 (17p13.1)
CHR17_8.49	RP11-12H18	0.39	0.55	0.97	2 genes: FLJ32734 (17p13.1) and MYH10 (17p13)
CHR17_9.54	RP11-208F13	0.52	0.65	0.98	N/a
CHR17_10.15	RP11-401O9	0.39	0.61	0.93	2 genes: MYH8 (17p13.1) and MYH13 (17p13)
CHR17_12.02	RP11-471L13	0.44	0.60	1.05	MAP2K4 (17p11.2)

*The shaded row shows the CHR position of TP53 in relation to clones that are present in this array slide.

Appendix 4 – CHR18

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR18_6.84	RP11-109E24	1.29	1.30	1.00	ATP8A1 (4p14-p12), problem clone – mapped entirely to CHR 4
CHR18_30.51	RP11-19F9	0.72	1.01	1.01	N/a
CHR18_35.34	RP11-108G3	0.72	1.01	0.79	N/a; Problem clone – mapped to CHR 11 and -X
CHR18_54.39	RP11-383D22	1.29	1.04	0.97	WDR7 (18q21.1-q22)
CHR18_75.59	**RP11-16L7	0.93	1.03	1.02	N/a; Problem clone all STSs mapped to CHR7

Appendix 4 – CHR19

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR19_8.58	CTD-2547N9	0.72	1.19	0.84	N/a
CHR19_36.92	CTC-416D1	0.69	1.06	0.91	N/a
CHR19_52.98	CTD-2571L23	0.68	1.12	0.88	5 genes, FLJ40321 mapped at 19q13.32, the others at 19q13.3 (CRX, GLTSCR2, EHD2, SEPW1)
CHR19_58.14	RP11-44L20	1.28	1.19	1.09	N/a; mapped to 19q13.41b
CHR19_59.39	CTD-2337J16	1.21	1.14	1.07	3 genes all mapped to 19q13.4 (LILRB5, LILRB3, ILT8)
CHR19_59.94	RP5-1060P11	1.32	1.30	0.93	7 genes: KIR2DL5 mapped to 19p13.3, and 6 remaining all clustered at 19q13.4 (KIR2DL2, KIR2DL4, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5)

Appendix 4 – CHR22

CHR position (Mb)	Clone	Fluorescence ratio (Mean) Fluorescence Ratios (Test/Ref) 1612	1724	CTRL	Matching genes (locus)
CHR22_21.47	RP11-50L23	0.71	0.78	1.07	N/a; Reported as "Redundant"
CHR22_24.21	CTA-390B3	0.69	1.04	0.83	MFNG (22q12)

Appendix 5 - Sample list (N=135) of known polymorphic and "dodgy" clones

Cancer_2B6	NONSC6D8	RP11-444B5
CTB-31C16	NONSC6E9	RP11-446P9
CTC-490G23	NONSC7A3	RP11-451G1
CTC-820M16	NONSC7D6	RP11-455J20
CTC-963K6	NONSC7E4	RP11-457E6
CTD-2337J16	NONSC8D3 = RP11-209H16	RP11-46C20
NONSC12A12	NONSC8F3	RP11-478K18
NONSC12A3	NONSC9D5	RP11-484P7
NONSC14A5 = RP11-462G12	NONSC9D6	RP11-489K4
NONSC15G12	RP11-105C19	RP11-497H16
NONSC16C6	RP11-10N17	RP11-505G12
NONSC16D7	RP11-114O8	RP11-52C8
NONSC1A3	RP11-11N15	RP11-543C4
NONSC24A3 = RP1-29O12	RP11-11N16	RP11-551B22
NONSC24D1	RP11-12D3	RP11-557N21
NONSC27A1	RP11-13E1	RP11-6C14
NONSC29B9	RP11-142A12	RP1-171F15
NONSC29E10	RP11-144K9	RP1-179P12
NONSC2B11	RP11-147L13	RP11-84H6
NONSC2H4	RP11-164L18	RP11-87C12
NONSC32E12	RP11-209H16	RP11-94E2
NONSC32E8	RP11-220N20	RP11-96B2
NONSC32G1	RP11-229G4	RP1-215P15
nonsc35B9	RP11-23N11	RP1-29O12
nonsc35F4	RP11-23N2	RP1-93N13
nonsc35H1	RP11-24H13	RP5-1060P11
nonsc35H6	RP11-24I19	RP5-1108M17
nonsc38B3	RP11-25F15	RP5-1109K10
NONSC3A10	RP11-270H4	SC1_1Mb_BAC1A1
NONSC3B12	RP11-279F15	SC1_1Mb_BAC2C10
nonsc40A2	RP11-285F23	SC10BAC-1Mbset-1C5
nonsc40E5	RP11-292F22	SC20BAC_1Mb_setA4
nonsc40F10	RP11-296I10	SC20BAC_1Mb_setB2
nonsc41A3	RP11-2F9	SC20PAC_1Mb_setD3
nonsc41F1	RP11-305D15	SC6BAC1Mbset1A6
nonsc43D9	RP11-312H1	SC6BAC1Mbset1B8
NONSC4C12	RP11-316G9	SC6PAC1Mbset1B4
NONSC4E7	RP11-324I10	SC9BACMbset_1E9
NONSC4F4	RP11-326G21	telomereA1
NONSC5C8	RP11-328M22	telomereB2
NONSC5E12	RP11-32C20	telomereB3
NONSC5G8	RP11-368N21	telomereC1
NONSC5H5	RP11-374N3	telomereC4
NONSC6A6	RP11-386L3	telomereG2
NONSC6D5	RP11-424O11	telomereG4

APPENDIX 6: ABBREVIATIONS

~	An estimate, 'approximation'
>	More than
3' UTR	3 prime untranslated region
A148T	Alanine to threonine substitution at codon 148
A2B5	Monoclonal antibody
AA/C	Anaplastic astrocytoma/cell culture (sample)
AA/S	Anaplastic astrocytoma/solid (sample) or biopsy
AAs	Anaplastic astrocytoma(s)
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
ABI1	Abl-interactor 1
ABL1	V-abl, Abelson murine leukaemia viral oncogene homolog 1
Add/add	Added (in a translocation)
AKT	V-akt murine thymoma viral oncogene homolog (1, 2 and 3)
ALL	Acute lymphocytic leukaemia
ANKRD22	Ankyrin repeat domain containing 22
APC	Adenomatosis polyposis coli
ARID3A	AT rich interactive domain 3A BRIGHT-like
ARL6	ADP-ribosylation factor 6
ARL7	ADP-ribosylation factor 7
ARPs	Astrocyte restricted precursor
AS	Angelman syndrome
ASCL2	Achaete-Scute complex homolog-like 2 family member
ASCL3	Achaete-scute complex like 3
ASTN/ASTN1, -2	Astrotactins/astrotactin 1, -2
ATRNL1	Attractin-like 1
AXIN1	Axin1 (a negative regulator of the wingless-type member 1
BA	Brodmann areas
BAC	Bacterial active chromosome
BCAS4L	Breast carcinoma amplified sequence 4 like
BCAS4	Breast carcinoma amplified sequence 4
BCR	Chromosome 22 breakpoint cluster region
BHLH	Basic helix-loop-helix

BIN1	Encoding bridging integrator 1 (initially identified as a MYC-interacting protein with features of a tumor suppressor).
BLBP	Brain lipid binding protein
BMP4	Bone morphogenetic protein 4
BMP5/BMP5.1	Bone morphogenetic protein 5
BMP8B	Bone morphogenetic protein, member 8B
BNGF	Nerve growth factor
bp	Base pair(s)
BRAP	BRCA1 associated protein
BRCA1	Encoding breast cancer 1, early onset.
BRCA2	Encoding breast cancer 2, early onset.
C9orf157	Chromosome 9 open reading frame 157
C9orf96	Chromosome 9 open reading frame 96
CCNC	Cyclin C
CCND	Cyclin D
CCND1	Cyclin D1
CCNE	Cyclin E
CCNE2	Cyclin E 2
CCNH	Cyclin H
CD44	CD44 antigen (homing function and Indian blood group system)
CDC10	Cell division cycle 10
CDC25A	Cell division cycle 25A
CDC25BandC20orf29	Cell division cycle 25B and chromosome 20 open reading frame 29
CDC25C	Cell division cycle 25C
CDC42	Cell division cycle 42
CDC42BPB	CDC42 binding protein B
CDK10	Cyclin-dependent kinase (CDC2-like) 10
CDK11	Cyclin dependent kinase (CDC2-like) 11
CDK2	Cyclin dependent kinase 2
CDK3	Cyclin dependent kinase 3
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6

CDKN1B	Cyclin dependent kinase 1B/p27/KIP1
CDKN2AandCDKN2B	Cyclin-dependent kinase 2A and cyclin-dependent kinase 2B
CDKN2D	Cyclin dependent kinase 2D
CDKs	Cyclin-dependent kinases
cDNA	Cmplementary DNA (originally made from RNA transcript)
CFLP1	Cofilin pseudogene
CGH	Comparative genomic hybridisation
CHD6/CHD5	Chromodomain DNA binding protein 6
CHEK2	Checkpoint homolog 2
CHR	Chromosome
CIN	Chromosomal instability
CKI	CDK inhibitor
CKIT/c-KIT/c-Kit	C-kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog)
CLK	CDC-like kinase 1
C-MET	Cellular MET (gene)
CML	Chronic myeloid leukaemia
CMT1A	Charcot-Marie-Tooth disease types 1A
CMT1B	Charcot-Marie-Tooth disease types 1AB
C-MYC	Cellular MYC
CNAs	(DNA) copy number aberration(s) (or alteration(s)
CNS	Central nervous system
COR	Cut off range
Cot-1	Human placental DNA, used to suppress cross hybridisation to repetitive DNA
CpG	Cytosine and guanine enriched regions in a chromosome
CRABP/CRABP1	Cellular retinoic acid binding protein/CRBP1
CRDs	Common regions of deletion
CSE1L	Chromosome segregation 1-like
CSF	Cerebrospinal fluid
CSPG4/NG2	Chondroitin sulfate proteoglycan 4/neurogranin
CT	Computed tomography

C-terminal	Carboxyl-terminal
CTNNB1	Catenin B1
DAPI	4,6-diamidino-phenylindole
dATP	Deoxyadenine triphosphate
DBSs	DNA-binding sites
DBX2	Encoding developing brain homeobox 2.
DCC	Deleted in colon cancer
DCC.1	Deleted in colon cancer
DCC/DCC.1	Deleted in colon cancer genes
dCTP	Deoxycytosine triphosphate
DDX1	DEAD (Asp-Glu-Ala-Asp) box polypeptide
DDX26	DEAD box protein
del	Deletion
dGTP	Deoxyguanine triphosphaste
DMBT1	Deleted in malignant brain tumours-1
DMRTA1	Methylthioadenosine phosphorylase (MTAP) and DBRT-like 1
DMs	Double minute(s)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside(s) triphosphate(s)
DOP-PCR	Degenerate oligonucleotide primed PCR
DPP	Decapentaplegic
dTTP	Deoxytymine triphosphate
DTX3L/BBAP	Deltex 3-like or B-lymphoma- and BAL-associated protein
dUTP	Deoxyuracil triphosphate
E1/E2	Hypothetical end-point in developmental pathways of astrocytomas
E2F	Eukaryotic transcription initiation factors, member 2
E2F1	Transcription factor E2F1
E2F2	E2F transcription factor 2
E2F5	E2F5 transcription factor
EcoRI	(Strain of) escherichia coli (restriction enzyme) 1
EDTA	Ethylene-diamino-tetra-acetic acid (anticoagulant, protease inhibitor, inhibits PCR reaction, washing solution

EFNA	Ephrin-A
EFNA2	Ephrin-A2
EFNA5	Ephrin-A5
EFNB3	EphrinB3
EGF/EGFR	Epidermal growth factor /receptor
EIF4	Eukaryotic translation initiation factor 4
EIF4A1	Eukaryotic initiation factor 4, A1
EIF4A-II	Eukaryotic transcription initiation factors
ELAV2	Embryonic lethal, abnormal vision 2
EMCN	Endomucin-like sialoprotein
EMX2	Mammalian homolog of empty spiracles 2
EN1	Engrailed
EN2	Engrailed-2
ENCR	European Network of Cancer Registries
ENO2	Neural enolase 2 gamma
EPH3	Ephrin receptor 3
EPHA7	Ephrin A7
ERBB2/Erbb2	V-erb-b erythroblastic leukaemia viral oncogene homolog 2
ERBB4	V-erb-a erythroblastic leukaemia viral oncogene homolog 4
ERPs	Ependymal restricted cells
ESP10	Hypothetical protein LOC441583.1 (similar to radixin
ESR	Estrogen receptor 1
Et OH	Ethyl alcohol
F-10 (HAM),	Nutrient Mixture
FCS	Fetal calf serum
FES	Feline sarcoma viral homolog
FGF	Fibroblast growth factor
FGFR3	Fibroblast growth factor receptor 3
Fgfr3-	Fibroblast growth factor receptor type three (negative)
Fgfr3+)	Fibroblast growth factor receptor type three (positive)
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
FIBCD1	Fibrinogen C domain containing 1
FIG	Fused in glioma

FISH	Fluorescence(or fluorescence) in situ hybridisation
FLJ10385	Codes for similar to WDR79 domain containing
FLJ23311	E2F–related hypothetical protein
FLJ40296	Hypothetical protein FLJ40296
FLJ45721	Codes hypothetical protein (often of the same name!)
FLJ46675	Codes for similar to dynein
FRA16D	Fragile site
FRA3B	Fragile site 3B
FRA7G	Fragile site 7G
FST	Follistatin
FRP	Fluorescence ratio profile(s)
G0	Growth arrest (resting) phase of mitotic cell cycle
G1	Resting state (phase)
G2	Second growth
GAB1	G-protein receptor binding 2 associated binding protein 1
GAPD	Glyceraldehydes-3-phosphate dehydrogenase
GBAS	Glioblastoma amplified sequence
GBMs	Glioblastoma multiforme(s)
GBX2	Homeobox gene (a vasoactive intestinal peptide receptor)
GDP	Glycerophosphodiester phopsphodiesterase domain containing
GFAP	Glial fibrillary acidic protein
GFOCs	Gliofibrillary oligodendrocytes
GFPs	GTPase-fusion proteins
GLI	Glioma-associated oncogene homolog 1
GLTSCR1	Glioma tumour suppressor candidate region, genes 1
GLTSCR2	Glioma tumour suppressor candidate region, genes 2
GOIs	Gene(s) of interest
Grb2	Encoding growth factor receptor-bound protein 2.
GRIK1	Glutamate receptor, ionotrophic, kinase 1
GRN	Granulin
GRPs	Glial restricted precursors
GTF2H2	General transcription factor II, polypeptide 2
GTF2H3	General transcription factor 2 H, polypeptide 3

GTP	Guanine triphosphaste
GTPase	GTPase activating protein
GUK1	Guanylate kinase 1
Gy	Gray (unit of radiation)
H & E	Haematoxyline and eosin
H2B	H2B histone family, member S
HBSS	Hank's Balanced Salts
HCMV	Human cytomegalovirus
HDAC4	Histone deacetylase C4 (a component of the p53-dependent DNA-damage response)
HDACs	Histone deacetylases
HDLBP	High-density lipoprotein binding protein
HES7	Hairy/enhancer of split, homolog 7
HEY2	Hairy/enhancer of split related with YRPW motif 2
HGAs	High-grade astrocytomas
HGF/SF	Hepatocyte growth factor/scatter factor
HLH	Helix-loop-helix
HMG	High malignancy grade
HMGAs	High malignancy grade astrocytomas
HNPCC1	Hereditary nonpolyposis colorectal cancer type 1
HNPCC2	Hereditary nonpolyposis colorectal cancer type 2
HNPP	Hereditary neuropathies with liability to pressure palsies
HOXA	Homeobox genes
HOXA1	Homeobox A1
HOXA13	Homeobox A13
HOXB	Homeobox class B genes
HOXD	Homeobox D
HPE	Holoprosencephaly
HRAS	Harvey rat sarcoma viral oncogene homolog
H-ras	Ras-viral oncogene homolog
HSR	Homogeneous staining region
hTERT	Catalytic subunit of the telomerase enzyme
IARC	International Agency for Research on Cancer

ICD-O	International classification of disease for oncology
ICH	Institute of Child Health
IDs	Imprinting defects
ids.(image format)	File format for storage and processing of image data using QUIPs software
IFI35	Interferon induced protein 35
IFITM1	Interferon induced transmembrane protein 1
IFNB1	Interferon, beta polypeptide I
IGF	Insulin growth factor
IGFBP2	Insulin-like growth factor receptor binding protein 2
IGFBP5	Insulin-like growth factor binding protein 5
Igl	Immunoglobulin light chain, lambda
IPF1	Insulin promoter factor 1
IPW	Imprinted gene in the PWS region gene
IRS3	HRBL, encoding HIV-1 Rev binding protein-like.
ISH	*In situ* hybridisation
ISL1	Motor neuron-specific Islet-1 transcription factors
Kb	Kilo (10^3) bases (nucleoside)
KCNIP4	Potassium voltage-gated (Kv) channel interacting protein 4
Ki-67/MIB	MK167, encodes antigen identified by nomoclonal antibody Ki-67
KPS	Karnofsky performance score
KREMEN1	Kringle containing transmembrane protein 1
KRT8	Keratin 8
LATS 2	Large tumour suppressor, homolog 2
LDHA	Lactate dehydrogenase A
LGA	Low-grade astrocytoma(s)
LGI1	Leucine-rich gene glioma activated-1
LIMK2	Encdes receptors for WNT/catenin-signaling pathways
LMGAs	Low malignancy grade astrocytomas
LN2	Liquid nitrogen
LOC440374	Encoding similar to RAB43, member of Ras oncogene family
Log (log)	Logarithm

LOH	Loss of heterozygosity
LRP	Low density lipoprotein-related protein 1
LTSs	Long-term survivors
M (phase)	Mitosis
MAGEL2	Melanoma antigen-like gene 2
MAP2K6	Mitogen-activated protein kinase kinase 6
MAP3K	Mitogen-activated protein kinase kinase kinase 5
MAP3K6	Mitogen-activated protein kinase kinase kinase 6
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
MAP4K2	Mitogen-activated protein kinase kinase kinase kinase 2
MAP4K5	Mitogen-activated protein kinase kinase kinase kinase 5
MAPKAP5	Mitogen-activated protein kinase-activated protein kinase 5
MB (Mb)	Million bases
MC4-R	Melanocortin 4 receptor
mCGH	Metaphase comparative genomic hybridisation
MCRD	Minimum common regions of deletion
MCRO	Minimum common regions of overlap
MDA	Multiple displacement amplification
MDM2	Murine double minute 2
MESP1	Mesodermal posterior 1
MFISH	Multi-colour/multiplex fluorescent/fluorescence in situ hyb.
MGC17330	Hepatocyte growth factor-like
MGMT	O-6-methylguanine DNA methyl transferase
MGST1	Microsomal glutathione S-transferase 1
MIF	Macrophage migration inhibitory factor
MIN	Microsatellite instability
MIST	Mast cell immmunoreceptor transducer
MKRN3	Makorin ring finger protein
Ml (ml)	Millilitre
µg	Microgram
µl	Licrolitre
MLCK	Myosin light chain kinase

MLH3	MUTL homolog 3
MM (mM)	Millimolar
MMR	Mismatch repair
MNOPs	Motorneuron-oligodendrocyte precursors
MPAG	Malignant primary adult-onset glioma
MPE600	Breast cancer cell line of known cytogenetic abnormalities, supplied by Vysis and used as positive control in CGH experiments
MRC	Medical research council
MRC BRO5	Multi-centre clin. trial (MRC Brain Tumour Working Party, 2001
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MRPL28	Mitochondrial ribosomal protein L28
MSH1	MutL homolog 1
MSH2	MutS homolog 2
MSH2	Muscle segment homeobox homolog 2
MSX1	Muscle segment homeobox 1 homolog
MTAP	Methylthioadenosine phosphorylase
mTOR	Mammalian target of rapamycin
MYBL2	V-myb, the myeloblastosis viral oncogene related (avian)-like 2
MYCN	V-myc myelobl. viral oncogene related, neuroblastoma derived
MYOD1	Myogenic factor 3
NBCCS	Nevoid basal cell syndrome
NCBI	National Centre for Bio Informatics (USA)
NEO1	Neogenin homolog 1
NEOROD	Neurogenin
NEUROD1	Neurogenin differentiation 1
NEUROD2	Neurogenic differentiation factor 2
NEUROG	Neurogenin
NEUROG1	Neurogenin 1
NF1	Neurofibromatosis 1 (gene)
NF2	Neurofibromatosis 2 (bilateral acoustic neuroma)

ng	Nanogram (10^{-9} grams)
NHLH	Nascient helix-loop-helix
NHLH2	Nascient helix-loop-helix 2
NHS	National health service
NID1	Entactin or nidogen
NIN	Nucleotide instability
Nkx2.2	NK2 related transcription factor, members 2
NKX2-9	NK2 transcription factor related, locus 9
NKX3-1	NK3 transcription initiation factor, locus 1
NKX3-2	NK3 transcription factor related, locus 2
NKX6-1	NK6 transcription factor related, locus 1
NKXX2.2	NK2 transcription factor related, locus 2
NMBR	Neuromedin B receptor
NME3/DR-nm23	Non-metastatic cell 3
NME4andDECR2	NME23-H4, encoding non-metastatic cells 4, protein expressed in
NMI	N-myc and STAT interactor
NNMT	Nicotinamide N-methyltransferase
NOG	Noggin
NP-40	Nonindet P-40 (cell lysis buffer)
NPPB	Natriuretic peptide precursor B
NRG1	Neuregulin 1
NRP	Neuron restricted precursors
NRP1	Neuropilin 1
NSC	Neural stem cell
Nt	Nucleotide
NTF3	Neurotrophin 3
NTF5	Neurotrophin 5
N-WASP	Neural Wiscott-Aldrich Syndrome
N-WASP/WASL	Wiscott-Aldrich syndrome-like
NXT1	Neurotrophin factor 2 (NFT2) – like export factor 1
O2A	Oligodendrocyte type-2 astrocyte precursors
OLIG2	Oligodendrocyte lineage transcription factor 2
OLIG3	Oligodendrocyte transcription factor 3

p16INK4A, p14ARF	Alias of CDKN2AandCDKN2B
p27KIP1/p27	CDKN1B, encoding cyclin-dependent kinase inhibitor 1B (Kip1)
p53	P53 protein
PAHs	Polyaromatic hydrocarbons
p-arm	The short arm of a chromosome
PAX2	Paired box 2
PAX3	Paired box 3
PAX6	Paired box 6
PAX7	Paired box 7
PBS buffer	Phosphate-buffered-saline
PC4	Activated RNA polymerase II transcription factor 4
PCR	Polymerase chain reaction
PCV	Procabazine, cisplatin, and vincristine
PDCD1	Programmed cell death gene
PDGF	Platelet derived growth factor
PDGFC	Platelet derived growth factor C
PDGFR	Platelet derived growth factor receptor
PDGFRA	Platelet derived growth factor receptor-a
PER7	Peroxismal biogenesis factor 7
PFS	Progression-free survival
PGY1	P-glycoprotein 1
PHKG1	Phosphorylase kinase gamma 1
PI3K	Phosphoinositide-3-kinase (3 catalytic subunits, alpha/beta/gamma
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PKD1	Encoding polycystic kidney disease 1 (autosomal dominant)
PMS1	Postmeiotic segregation increased 1
PMS2	Postmeiotic segregation increased 2
PP2A	Protein phosphatase 2A
pRB	Retinoblastoma protein
PRG2andSLC43A3	For proteiglycan 2 and solute carrier 43, member 3 polypeptide
PROM1	Prominin 1
PTCH	Human homolog of patched

PTEN/MMAC1	Phosphatase and tensin homolog/mutated in multiple adeno Ca 1
PTN	Pleiotrophin
PTPN1	Protein tyrosine phosphatase, non-receptor type 1
PVCs	Polyvinyl chrlorides
PWS	Prader-Willi syndrome
PWS/AS-SRO	PWS/Angelman syndrome-smallest region of overlap
QALY	Quality adjusted life year
q-arm	The long arm of a chromosome
QIAgen	Brand-name of a scientific company manufacturing 'quantitative image appliances'
QUIPS	Quantitative Image Processing System
RAC1	Encoding ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
Raf1	Encoding v-raf-1 murine leukaemia viral oncogene homolog 1
RARB	Retinoic acid receptor, beta
Ras	Member of Ras oncogene family
Ras-GTP	Ras-GTPase activating protein SH3 domain-binding protein
RB1	Retinoblastoma 1
RBL1 (p107)	Retinoblastoma like 1
RBL2 (p130)	Retinoblastoma like 2
RC2	RGN, encoding regucalcin (senescence marker protein-30)
Rheb	Ras homolog enriched in brain
RNA	Ribonucleic acid
ROBO1	Roundabout, homolog 1
RP11-61G19	BAC clone
RT	Radiotherapy
RT	Room temperature
RTK	Receptor tyrosine kinase
RUNX1 and RUNX1.1	Runt-related transcription factor 1 (acute myeloid leukaemia 1)
S	Synthesis (as of DNA in mitotic phase of a cell cycle)
S-100b	S100 calcium binding protein, beta (neural)
S-100b	S100 calcium binding protein beta (neural)

SAS	Sarcoma amplified sequence
SCA1/ATXN	Spinocerebellar ataxia/ataxin
SCX/bHLH.2	Scleraxis, the basic helix loop helix dimerisation region 2
SEER	Surveillance, Epidemiology and End Results
SEGA	Subependymal giant cell ependymomas
SEMA5A	Semaphorin
SERPINE/GDNPF	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2, or glial-derived neurite promoting factor, protease inhibitor 7
SERTAD1	SERTA domain containing 1
SGZ	Subgranular zone
Shc	Encoding `Src homology 2 domain containing transforming prot.
SHH	Sonic hedgehog
SIM1	Single-minded homolog 1
SKY	Spectral karyotyping
SLC1A1	Solute carrier family 1, member 1
SLC2A3/GLUT-3	Solute carrier family 2 (facilitated glucose transporter), member 3
SLIT1	Slit homolog 1
SLIT2	Slit homolog 2
SLIT3	Slit 3
SMAD1	Mothers against decapentaplegic homolog 1
SMAD6	Mothers against decapentaplegic homolog 6
SMAD7	Mothers against decapentaplegic (DPP) homolog 7
SMADs	Mothers against decapentaplegic (DPP) homologs
SMO	Smothered
SMOX	Hypothetical gene which codes for polyamide oxidases (Wang et al., 2003) that may have roles in cell proliferation and survival
SNARE	Similar to RIKEN cDNA A030009B12 gene.
SNURF-SNRPN	Small ribonucleoproteins-N-upstream reading frame-small ribonucleoproteins-N
Sos	Son of sevenless homolog
SOX12/SOX20	SRY-BOX 12

SPARC	AADH, secreted protein, acidic, cysteine-rich
SSC	Buffer made of sodium chloride and sodium citrate
SUFU	Suppressor of fused
SV	Subventricular
SV40	Simian virus 40
SV40 ER	SV40 early region
SV40T	Simian virus 40 large-T (oncoprotein)
SYM1	Symphalangism 1
SYNS1	Synostoses 1
T/t	Translocation
TAE	Tris-acetate-EDTA electrophoresis buffer
TCF21	Transcription factor 21 gene
TDGF1	Teratocarcinoma-derived growth factor 1
TFIIH1	Transcription factor 2H, member 1
TGF-b/TGFB	Transforming growth factor (ligand) beta
TGFR-beta	Transforming growth factor receptor-beta
TIF1	Thyroid nuclear factor / thyroid transcription factor 1
TIMP3	Tissue inhibitors of metalloproteinase 3
TNFAIP3	TNF alpha-induced protein 3
TNFSFII/OPGL	TNF (ligand) superfamily, member 11/osteoporin
TOR	Target of rapamycin
TP53	Tumour protein p53
TP53BP1	Tumour protein p53 binding protein, 1
TP73	Tumour protein 73
TSC1	Tuberous sclerosis 1 (merlin) (gene)
TSC2	Tuberous sclerosis 2 (tuberin) (gene)
TSGs	Tumour suppressor genes
TYRO3	Tyrosine-protein kinase receptor TYRO3 precursor
UBE3A	Ubiquitin 3E
UCL	University College London
UNC-40	Uncoordinated (centrosome movement)-40 (deleted in colon Ca)
UNC-6	Uncoordinated (centrosome movement)-6
UPD	Uniparental disomy

UV	Ultra violet (light source)
V	Volts
V-AKT/AKT1	Human homolog of the murine thymoma viral oncogene
VAX1	Ventral anterior homeobox 1
VEGF/ -alpha / -beta	Vascular endothelial growth factor/ -alpha / -beta
VEGFR/KDR	Human homolog of the proto-oncogene c-kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog, and vascular endothelial growth factor receptor or kinase insert domain receptor
VZ	Ventricular zone
WDR79	WD repeat domain 79
WHO	World Health Organisation
WHO grade I	Pilocytic astrocytomas
WHO grade II	Astrocytoma
WMPCs	White matter precursor cells
WNT	Wingless type
WNT2B	Wingless-type MMTV integration site, member 2B
WRDandSH3BGR	SH3 domain binding glutamic acid rich protein
XBF-2	Xenopus brain factor 2
ZNF33A	Zinc finger protein 33A

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		Metaphase CGH data	
	Tumour ID	Gains	Losses
UNKNOWN GLIOBLASTOMAS	S11	None	1p36.3-p36.1; 16p13.3-p13.2; 16q11.1-q11.2; 16q24; 17p13; 17q24-q25; 19p13.3-p12; 19q13.3-q13.2; 22q13; Xp22.3-q28
	GBM/S3044	1p33-p13, 1q24-q31, 2q22-q24, 2q31-q32, 3q24-q25, 4p15.1-p14; 4q13-q32; 5q13; 5q14-q23; 6q11-q22; 7p21-p12; 7q21; 7q22-q31; 11q14; 12q15-q21; 12q24.1-q24.3; 18q11.2-q12	1p36.3-p33; 2q37; 9q12, 9q34; 10q25-q26; 13q34; 14q31-q32; 15q11.2; 16q11.2; 17p13-p12; 17q24-q25; 19p13.2-q13.3; 20q13.2; 22q11.2-q13; Xp22.3-q21; Xq21-q28
	GBM/S2848/mda*	Xp22.3-q28	None
	GBM/C1397	1p22; 3p12-p11; 4q22-q24; 4q26-q27; 5q14-q22; 9p24; 13q21-q31	1p36.2-p32; 19p13.3-q13.42; 22p13-q13; Xp22.2-q27
	GBM/1719/mda	2p11.2-q11.2; 2p25-p24; 3p12-p11; 3p26-p25; 3q28; 4p16; 4q11-q12; 4q34-q35; 5p15.3; 6p25; 6q22; 8p23; 8q24.2-q24.3; 9p24-p23, 10p15; 11p15; 11q24; 12p13; 12q24.3; 13q34; 18p11.3; 18q22-q23; 20p13; 21q22	1p35-p34.3; 9q33-q34; 16p13.2-p11.2; 17p13- q24, 19p13.3-p12; 19q12-q13.3; 22q11.2-q13; Xp22.1-p21
	GBM/S1575	1p31-p22; 2q21-q24; 2q37; 7p21-q11.2; 7q21-q32; 12q13-q21; 18p11.3	1q12; 9q11-q13; 10p11.2-q11.2; 10q21; 10q23; 10q26; 13q11; 15q11.2; 16p13.3-p13.2; 16q11.1-q11.2
1° GBM	GBM/C2394	Xp22.2-q27	None
	GBM/C2685	None	15q11.2-q12

*, Tumours that had <3 CNAs (copy number alterations) in the autosomes (n=5).